

Attrition, not conquest, of disease

The AIDS saga has been a nasty shock for people accustomed to take modern medicine for granted. The discovery of the virus responsible should remind them of the enduring role of medical research.

AIDS (acquired immune deficiency syndrome) has engendered high drama from the beginning, a mere three years ago. Not every day does a new disease entity pop up, apparently from nowhere. Nor does it often happen that the main foci of a new disease are not in some far-off and half-forgotten developing country but in the most industrially advanced communities in the world. AIDS has shaken the complacency of hosts of people in no present danger of becoming afflicted.

The relatively small numbers of those suffering overtly from AIDS (thousands in the United States, hundreds in Western Europe) have been counterbalanced in people's imaginations by the bizarre features of the condition. People almost always die, but from one of several secondary causes. Most patients have been homosexual men, but women and their newborn children as well as people receiving blood transfusions and factor VIII (in haemophilia treatment) have been affected. This has raised alarm that whatever agent may be responsible is confined to those now afflicted only fortuitously — and temporarily. The notion, increasingly plausible in recent months, that the agent responsible is most probably a virus has been particularly shocking for smug health-ridden societies long since persuaded that medicine is a routine technology, even a physicians' ramp, and that good health is either a consumer purchase or an attitude of mind. Was not infection abolished with penicillin, anxious people have been complaining?

The latest instalment of the AIDS drama should give the lie to many of these nonsensical expectations. AIDS is almost certainly caused by a virus, that most clearly characterized by Robert Gallo and his associates at the National Cancer Institute in the United States, but with a high chance that the virus previously described by Luc Montagnier at the Pasteur Institute in Paris is identical. Both are retroviruses carrying genetic information as RNA, a DNA version of which becomes incorporated into the genes of infected cells. The most telling evidence is that a majority of the patients with AIDS carry antibodies against one of the viral proteins — at some earlier stage, their immune systems have responded to an infection which has nevertheless overwhelmed a large proportion of them. But as yet, little is known of the mechanism of infection, of the reasons why one class of T cells among lymphocytes is put out of action by the virus or of the status of those who have already been infected (to judge from the antibodies they carry) but who have not developed symptoms of AIDS. (The haunting question is whether they are immune or destined to run into trouble.) And next to nothing is known of the natural history of the virus. So why, the sceptics will be asking, is there all this fuss? If neither Gallo nor Montagnier can offer a cure, what benefit is there in what they have accomplished?

Virus

The simplest answer is also primitive. AIDS, a mystery only a few weeks ago, has been transformed into another virus disease. And that transformation, even though it will not by itself prevent the death of a single patient, has the utmost practical importance. Correspondents this week (p.12) and last have explained what steps can now in principle be taken — the identification of those among the sexual partners of AIDS patients who have been exposed to the virus (and who may or may not be carriers or at risk themselves), the screening of transfusion blood (if practicable)

and so on. The essential tools for these investigations are the standard techniques of pre-molecular immunology. So AIDS has been lumped in the same category as, say, measles.

The same techniques will play a crucial part in helping to unravel the natural history of the disease (and the suspected link with central Africa) — as will the mere knowledge that a virus is indeed responsible. The only practical benefit certain to flow from what will now happen is that some people at risk will be helped to avoid infection. But the conversion of a mystery into a medical problem will also provide people with a sense of confidence that would otherwise not have been supportable. What has happened with AIDS is yet another illustration of the well-known truth that to know the cause of a problem is a necessary part of its solution.

Control

But when will there be a vaccine to prevent the spread of AIDS, or even a way of curing those already infected? That is what public health officials need, and what many of their constituents will insist can alone count as progress. The again maddeningly simple answer is that nobody can tell. It has taken fifteen years from the recognition of the then-surprising life-cycle of the hepatitis-B virus to the development of vaccines (now going into trials) whose efficacy is not yet known. AIDS is only the second human disease known to be associated with a retrovirus — the other is adult T-cell leukaemia, first recognized in south-east Japan — and the obvious ways of going about the job of vaccinating against AIDS could yet prove ineffective. The prospects for more effective treatment for patients already stricken are even less certain (although not necessarily more distant). But when the causes of death in AIDS are the accidental infections patients pick up and the otherwise rare skin cancer called Kaposi's sarcoma, and if the reasons for these developments are the known deficiency of helper T cells in patients' blood, the only guarantor of more effective treatment is the better understanding of the human immune system at which literally hundreds of laboratories have set their sights. (It will be sheer good luck if the trials with the genetically engineered humoral agent interleukin-2 also now being mounted turn out to be successful.) Like it or not, those who look for quick results must cultivate patience instead.

Those to whom that advice is unpalatable should reflect that things might be much worse. Montagnier's observations, reported last month (*The Lancet* i, 753; 1984), turned on the lucky accident that of two haemophilic brothers being treated with factor VIII, only one had developed overt AIDS when first seen. Gallo's investigation, based on samples from many more patients, was a natural extension of his group's interest going back for four years in human T-cell leukaemia virus, type 1 of which may or may not be the same as that responsible for adult T-cell leukaemia in Japan. Here again, however, it is good luck that Gallo's group is now well placed to apply to the further definition of the AIDS virus the techniques of genetic manipulation being vigorously applied to the understanding of the role of retroviruses and oncogenes in carcinogenesis. In other words, the understanding of AIDS that has now been wrung from a seemingly obdurate problem is striking proof of the value of the basic biomedical research on which people have for decades enthusiastically lavished their talents — and governments, less



In these terms, rapid demonstration that AIDS is indeed caused by a virus is a triumph, even if not quite the "triumph over disease" that Mrs Margaret Heckler, the US Secretary of Health, described it last week. The triumph is that the research community has been able to respond so quickly to an unexpected development such as the emergence of AIDS. The explanation is again simple, exasperatingly so — the richness and diversity of the biomedical research enterprise, in the United States in particular but also throughout the world.

More good luck? Almost certainly not. The chances are high that in the present pattern of medical research, there would have emerged some group of workers somewhere in the world able — and willing — to turn from its long-term interests to throw light on a medical emergency such as that which cropped up three years ago. This is exactly what happened a decade ago with Legionnaires' disease, in its time also a threat to people's peace of mind. None of this diminishes the importance of what Montagnier and Gallo have separately done although, with both Legionnaires' disease and AIDS, the work done by the Centers for Disease Control at Atlanta, Georgia, deserves widely to be applauded. (So too does that of the clinicians who, in the past three years, have been required to treat AIDS patients with forms of therapy offering palliation only, who have in the process courageously taken to unprecedented lengths the openness and compassion that ideally characterize relationships between physicians and their patients and whose services in this regard will unhappily be required for many years to come.)

The moral, for Mrs Heckler as well as for the impatient sections of advanced communities eager to indulge the luxury of pretending that the practice and the pursuit of medicine have long since been replaceable by suitable doses of orange juice, physical exercise and mental application, is that the deliberate provision of medical care is in one important sense only a little more certain now than, say, a century ago. By means of large and costly social expenditures in research laboratories and clinics, the incidence of avoidable death is kept at a socially acceptable low level, but there is no avoiding the hazards still unknown. The best that can be done is to be prepared to deal with these hazards quickly, as they declare themselves. The case of AIDS has triumphantly illustrated the versatility of the biomedical research system patiently put in place in recent decades. Successive US governments have been especially farsighted in recognizing the importance of such a development. It is to be hoped that this latest emergency will help to win wider acceptance of the value of a vigorous research enterprise. For there is no prospect of a triumph over disease in general.

Double-talk on animals

NIH seems more ready to risk its reputation than to meet serious critics on animal care.

ANTIVIVISECTIONISTS and other crusaders have never made intellectual integrity one of their top priorities. Appeals to emotion, however inconsistent or however specious, continue to be the hallmark of those who wish to halt animal experiments. So it is no accident that cute animals — preferably those with a lot of fur — star in the antivivisectionists' advertising campaigns (which have now reached the underground railway system in Washington, DC, in the form of posters of a child with a dog and a message that scientists who have perfectly reasonable alternatives to animal experimentation are hoping to make that dog suffer).

The National Institutes of Health (NIH), which support the majority of animal experiments in the United States, have now apparently decided that they too can play that game. At the first of what will be a series of meetings around the country, NIH produced for the public's heartstrings real-life patients and their families who have benefited from animal experiments. While the sincerity of what these patients had to say is indisputable, one has to feel uncomfortable at the exploitation of their misfortune inevitably at play when the federal government stands them up to

say how much they love their husbands and how grateful they are that medical research has kept their children's daddies alive.

One has to feel even more uncomfortable at the nagging suspicion that NIH's simultaneous announcement of a new and supposedly tougher set of rules for the care and use of laboratory animals is nothing more than a part of this same public relations campaign. This conclusion can only be reinforced by NIH's simultaneous release of a study of animal experimentation at ten research institutions, chosen at random, that finds the old rules are working fine and that there are no problems to correct. If so, why correct them, unless the aim is to make a — very public — point?

In reality, the new regulations, which are published as a proposal open for public comment, do very little. Research institutions under the new regime will not only have to "commit themselves to implementing" animal care requirements — they will now have to "implement" them, a major crackdown if there ever was one. The only substantive change in the proposal is that the institutional animal care committees, charged with monitoring the use of laboratory animals at each research institution, would have to include one outside member.

NIH director James Wyngaarden set the tone for the NIH stance when, at last month's meeting, he declared his "deep concern" that public support for laboratory experiments involving animals may be eroding, the result of "politically sophisticated critics" who are attacking animal experiments as unnecessary and inhumane. NIH arguably have a role in public education to counter this trend, but need not sacrifice integrity in the process. If "political sophistication" means issuing meaningless regulations for the sake of appearances, NIH are better off with naivety. Or is it that by invoking the bogey of rabid antivivisectionists seeking to shut down biomedical research, NIH hope to distract attention from some serious proposals for reform (as opposed to revolution)?

It taxes credulity to claim that the extreme "animal rights" positions inherent in the antivivisectionists' stance are about to sway the overwhelmingly carnivorous population of the United States, or indeed that they are more than a sterile pseudo-philosophical exercise. A much more serious threat to present practice is not the crazed antivivisectionists but, rather, some sensible criticisms from sensible people. Senator Robert Dole, for one, has introduced legislation that, while respecting the absolute freedom of researchers to design scientific protocols and to make their own judgements on the necessity of using animals, would clamp down on some of the more serious persisting abuses in the care of laboratory animals and their use. The bill would require the use of analgesics, tranquillizers and anaesthetics except when scientifically impossible, would forbid the use of the same animal in more than one major operative procedure and would require either proper postoperative care or prompt euthanasia. Enforcement would be left largely to the animal care committees, which would have to certify in regular reports that experimental procedures involving animals followed these requirements. Significantly, the bill does not limit the freedom of scientists to proceed with such experiments; it does, sensibly, require that investigators "consider" alternatives and consult with a veterinarian in planning procedures involving unanaesthetized animals.

There is hardly anything subversive about these proposals. (Nor is there anything subversive in demands of animal welfare activists, such as Mrs Christine Stevens of the Animal Welfare Institute, that inspections now required by federal law should actually be carried out to ensure that laboratory animal care meets the published guidelines — which means basic things like clean cages, proper food and proper ventilation.) The US Department of Agriculture, which is charged with carrying out the inspections, has never been given the funds to discharge its responsibilities under the law. Yet such inspections as it is able to carry out reveal continued problems in these basic necessities for humane care. NIH would do well to face up to these issues, rather than issuing pronouncements full of sound and fury that signify nothing.

Star wars

Pre-election Congress turning sour

Washington

THE militarization of space may yet become a significant issue in November's presidential election in the United States. For the first time, Mr Walter Mondale, the Democratic front-runner, last week promised to order a freeze on space weapons if he is elected President. In Congress, meanwhile, even the Republican Senate is alarmed about President Reagan's desire to press ahead with deployment of anti-satellite weapons and research on a "star wars" defence against nuclear missiles. Dr George Keyworth, the President's science adviser, was given a frosty reception when he told the Senate Foreign Relations Committee that he opposed negotiating a ban on space weapons lest it blocked the star wars programme.

Congressional anxiety can only have been heightened by the publication of a report by its own research arm, the Office of Technology Assessment (OTA), concluding that the prospect of creating a near-perfect defence is "so remote that it should not serve as the basis of public expectation or national policy". Although detailed criticisms of the star wars plan have been published before — most recently by the Union of Concerned Scientists — the new OTA study is the first to be published by a neutral body with full access to classified information. Its pessimistic conclusions are likely to have considerable influence on the mood of Congress.

The report, prepared by Dr Ashton Carter of the Massachusetts Institute of Technology, covers largely familiar territory by drawing attention to the difficulties of propagating directed energy weapons with the range and accuracy to knock out a large-scale nuclear attack, and pointing out that for every defence concept proposed so far, simpler and usually cheaper countermeasures have already been identified. One of the most forthright parts of the report, however, points to four common "misapprehensions" about the prospect for a perfect defence. They are:

- Confusing the successful development of single devices — lasers, mirrors, aiming mechanisms and the like — with the creation of a successful system. Multiplying by even a million some directed energy weapon will not necessarily give a perfect defence.

- Equating star wars with past technological challenges, such as the Moon landing or the Manhattan Project. The report notes a "vital difference" between working around constraints imposed by nature and competing, in the case of star

wars, with a hostile intelligence bent on sabotaging the effort.

- Hoping for a technological development that will dispel all difficulties; when perfection of the X-ray laser for example, might turn out to benefit offence more than defence.

- Expecting to know with reasonable

confidence how a complex defensive system would perform in reality. Unlike defences used in previous wars, this one would have no chance to learn or adapt.

OTA concedes that it might make sense to construct a star wars system even if it is less than perfect, in order, for example, to protect missile silos or simply to protect as many lives as possible. But it complains that, despite the fanfare with which President Reagan's star wars proposal was announced, its precise aims remain unclear. For that reason, explicit standards against which its technical feasibility can be measured have proved elusive.

Peter David

Genetic engineering

Genentech claims factor VIII

ONE of genetic engineering's glittering prizes was claimed last week by Genentech Inc., the San Francisco-based biotechnology company, working in collaboration with the Haemophilia Centre at the Royal Free Hospital Medical School, London, and Speywood Laboratories in Wales. The human blood protein known as factor VIII, which is lacking in haemophiliacs and essential for their treatment, has now been cloned and expressed in biologically active form in a mammalian cell culture. The availability of a completely sequenced cDNA clone for factor VIII, the largest protein ever produced through recombinant DNA technology, is an essential first step towards a source of the factor that is independent of supplies of human blood plasma and so not susceptible to contamination by viruses.

Most factor VIII used in Britain is prepared from concentrates that may contain the pooled plasma of up to 20,000 blood donors. All such concentrates are infected with hepatitis viruses and many haemophiliacs suffer from liver dysfunction caused by non-A and non-B hepatitis. Existing alternative production methods may lead to a lower risk of contamination but have their own disadvantages. Haemophiliacs are also a high risk group for acquired immune deficiency syndrome (AIDS), almost certainly because an AIDS virus can contaminate factor VIII produced from plasma. It has been clear for the past three years that any replacement of comparable efficacy would be assured of a huge market.

The Haemophilia Centre in London developed a method of obtaining factor VIII in high purity using monoclonal antibodies to the protein and established the gross structure. At Genentech, a genetic probe derived from a factor VIII subunit was used to test genomic libraries until a clone was identified that matched one possible predicted DNA sequence. The factor VIII gene turns out, as expected, to be located on the X chromosome. Eventually a clone of the complete gene was obtained (190 kilobases long) and a cDNA clone of

factor VIII produced. After transfection of the clone into a mammalian cell line (Genentech will not say what type), human factor VIII was detected in the culture medium and measured by a purified enzyme system. The activity of the material is quenched by monoclonal antibodies to human factor VIII — further evidence that factor VIII is being produced.

Genentech is at pains to point out that there are several years' more work ahead before factor VIII will be produced by genetic engineering on a commercial scale, though it hopes to produce enough factor VIII for clinical trials of the product (probably around 100 mg) within 2 years. The aim now will be to obtain a cell line able to sustain high output of the protein and which can also be cultured on a relatively large scale. Last week's announcement preceded any publication of the genetic discoveries in a refereed scientific journal, but both Genentech and the team at the Haemophilia Centre in London say that papers are planned.

Naturally, nothing is as yet known of the economics of factor VIII production by genetic manipulation. There are, however, about 20,000 users of factor VIII in the United States, and as many again in Western Europe. Supplying known needs would be worth sales of at least \$100 million a year. Knowing how to manipulate large molecules such as the components of the blood-clotting system will be worth much more to the company which first succeeds in making such materials.

The announcement last week will be a blow for several other biotechnology companies working on factor VIII. Previously, only parts of the gene had been sequenced. Genentech says it intends to protect fully the processes it has developed by patents, and a spokeswoman added ominously that the company had recently expanded its patent staff. But the usefulness of patents in protecting biotechnology processes generally is not yet established, and one disgruntled competitor summed up his feelings by saying "better late than never".

Tim Beardsley

Cancer research

Direct-mail medicine criticized

Washington

A NEW organization created to raise funds in the United States for studies of diet, nutrition and cancer is coming under fire for spending only a small proportion of the millions collected through direct-mail solicitations on actual research. The American Institute for Cancer Research, founded in 1981 by two professional fund-raisers in the Washington area, took in some \$3.5 million during its first fiscal year ending September 1983, while awarding only \$400,000 in research grants.

According to a financial disclosure filed with New York State on 16 April this year, \$600,000 was spent on public education and \$2.4 million on fund-raising. And a report issued last month by the Council of Better Business Bureaus (BBB), an independent group that monitors charities and investigates consumer complaints, revealed that \$893,000 of the institute's fund-raising expenditure went to the firm owned by the institute's founders, Jerry Watson and Chat Hughey.

The BBB report concluded that the institute failed to meet its standards for charitable solicitations, which require that at least half of the funds raised be applied to the programmes described in the organization's public appeals. The BBB standards also require that the organization should have an independent governing body.

Since the end of the fiscal year, the institute has made some changes to correct these deficiencies, notably by amending its by-laws to restrict the founders' rights to appoint or remove directors of the institute.

A spokeswoman for the institute said that New York State, which has the strictest disclosure laws in the country, included under "fund-raising" close to a million dollars in expenditures that the institute considers to be "public education" activities, such as the printing of newsletters. According to an audit commissioned by the institute itself, only 37 per cent of its receipts went on fund raising. The New York rules consider an activity such as mailing a newsletter to be fund-raising if it is combined with an appeal for funds and if fund-raising is the "primary purpose".

The direct-mail pieces that the institute is continuing to send out, however, have raised the hackles of many scientists, the American Cancer Society and, according to a report in the *Los Angeles Times*, the state attorney general's office in California. More than 11 million copies of a "Census on Diet and Cancer" were sent out, which combines on a single page questions such as "what vitamin supplements do you take?", "do you eat red meat at least three times a week?" and "have you ever had cancer?" with a "contri-

bution reply" that offers the opportunity to make a tax-deductible contribution. Respondents are asked to tick a box that reads: "I agree! It's absolutely essential to do extensive research in the area of diet and cancer — especially now when it's estimated that 50 per cent of all cancer is diet-related."

The institute's spring 1984 newsletter quotes letters from enthusiastic donors, including such comments as "unfortunately medical schools appear to completely overlook (*sic*) the importance of proper nutrition in maintaining good health" and "I firmly believe . . . that many illnesses can be prevented, halted, and even cured by proper diet".

Dr Colin Campbell, a Cornell University biochemistry professor who served on the National Academy of Sciences panel on diet and cancer, was named senior scientific adviser to the institute last December. He acknowledges that the "census" continues to make him "uncomfortable" and that it is of virtually no scientific value. He said he had told the institute staff that it was unacceptable but had rewritten the questions to make them "neutral" at least. He said he had also rewritten a cover letter to make it less "scary" and more positive in tone, and the institute's fund-raisers had agreed to try it, although he said they had told him they were not sure it would work. The original letter had said

"Cancer can strike you, your parents, your brothers and sisters — anyone".

Campbell said he had instituted a new process for reviewing grant applications modelled on that used by the National Institutes of Health study sections, and was taking steps to advertise the availability of grants. Until now, the availability of the funds had been spread only by word of mouth. Although the institute does not release the names of its seven-member review panel, a list obtained by *Nature* confirmed Campbell's assurances that they are all reputable research scientists.

Campbell said he felt it unfair to judge the institute harshly on its performance during its first year of operation. The spokeswoman said the institute hopes to disburse 20 per cent of its funds in research grants this year. And she cited as an example of the institute's activity in public education the mailing of 20,000 copies of the National Academy report to physicians and the distribution of one million cards on breast self-examination for women.

In addition to the \$400,000 in grants made last year, a further \$700,000 had been committed and is being paid out, according to Campbell. Campbell's new review panel met in March and approved a further nine research proposals. The largest recipients of the awards made last year were the Linus Pauling Institute (\$70,000), the American Health Foundation (\$50,000) and Theodore Krontiris of Tufts University (\$50,000). Three members of the scientific panel, including Campbell, have also received grants. **Stephen Budiansky**

NIH to bolt the stable door

Washington

A NEW study by the National Institutes of Health (NIH) has recommended tighter procedures at their clinical research centres to prevent a recurrence of the Darsee affair. Dr John Darsee, who had been widely regarded as a brilliant young medical researcher at both Emory University and later at Harvard University, was found to have repeatedly fabricated research results.

The NIH study of Darsee's activities at the Emory General Clinical Research Center, one of 75 such centres supported by NIH, concluded that trainees should be supervised more closely and that co-authors of scientific publications should be required to accept responsibility, in writing, for the papers.

The study offered no recommendations specifically for Emory, noting that the university had taken action on its own to prevent a repetition following its internal investigation of the Darsee affair. A major weakness cited by the NIH investigation was the absence of procedures at Emory at the time to detect Darsee's unauthorized inclusion of faculty members as co-authors on his many publications and abstracts — and the failure of even witting co-authors

to detect anything suspicious about Darsee's research results. The investigators were told that although the chairman of the department of medicine at Emory did routinely review all manuscripts produced by the department, his review was "primarily intended to insure that supporting grants and services are acknowledged correctly".

The recommendation for closer supervision of trainees arose from the investigators' discovery that the Emory faculty members with whom Darsee collaborated each assumed that another faculty member was supervising Darsee's clinical studies. The NIH study recommends the naming for each young investigator of a single supervisor who would be expected to review regularly the trainee's clinical studies, including raw data.

The NIH investigation was conducted by Drs Evelyn Hess (University of Cincinnati), Darryl DeVivo (Columbia/Presbyterian Medical Center) and James Freston (University of Connecticut). Its recommendations are now being considered by the NIH Division of Research Resources, which administers the clinical research centres.

Stephen Budiansky

Agent Orange

Veterans' case comes to court

Washington

THE complex saga of Agent Orange, 11 million gallons of which were sprayed on Vietnam between 1962 and 1971, will finally come to trial before a federal court in Brooklyn, New York, on 7 May. A jury will have to decide whether the chronic health problems of nine plaintiffs, including five veterans and three children born with catastrophic birth defects, were caused by exposure to dioxin contaminant tetrachlorodibenzodioxin (TCDD) found in the herbicide. A huge amount of money is at stake; the plaintiffs represent the entire class of veterans and families who attribute medical problems to exposure to Agent Orange. Damages awarded against the companies concerned could amount to thousands of millions of dollars.

Although the class action is directed principally against seven manufacturers of Agent Orange, the federal government is expected to be in court too — very much against its will. The manufacturers, claiming that the federal government knew about the possible health effects of the defoliant when it used it, have filed third-party complaints that could force the government to share in any liability. The courts have so far rejected the government's claim that it cannot be sued for injuries received during military service.

The question of who knew what about Agent Orange, and when, will be a central issue in the trial. One of the allegations made against the manufacturers is that one of them, the Dow Chemical Company, was worried about the toxicity of dioxin as early as 1965, but tried to hide its concern from the federal government. Dow vigorously denies the charge. It says its worries in 1965 concerned the exposure of its employees to large concentrations of TCDD during manufacture, not use of the finished product which contained vastly lower concentrations. It also maintains that the Department of Defense knew at least as much as the manufacturers about the components of Agent Orange and the toxicity of TCDD.

But Dow and its co-defendants intend to base their defence on scientific arguments. They maintain that not enough research has yet been done to conclude whether the health of Vietnam veterans and their families differs significantly from that of the rest of the population. The evidence that is available, they argue, suggests that Agent Orange could not have caused the health problems ascribed to it.

Information about the effect of TCDD on humans is indeed sparse, although Congress has occasionally rapped the American Medical Association over the knuckles for saying so. Most of the data available have come from studies of industrial accidents, where victims are typically subjected to high exposures for

short periods, not to low exposures over a long period as in the case of troops in Vietnam. Even so, the only TCDD-related disorder about which there is no argument is chloracne, the skin disease.

Preliminary studies of the impact of Agent Orange have so far failed to establish a clear link between the herbicide and the many disorders — including birth defects, lymphoma, neuromuscular weakness and liver enlargement — for which it has been blamed by veterans. The scientific evidence has been further muddled by the findings of the Air Force's own epidemiological study of the personnel involved in the "Ranch Hand" operation, as the herbicide-spraying operation was called. The Air Force reported in March that there was little evidence that the Ranch Handlers had suffered as a result of contact with Agent Orange. None had soft-tissue sarcomas or chloracne, although the incidence of certain birth defects, skin cancer and liver disorders was high.

Like many other studies of Agent Orange, the Ranch Hand results have been hailed as a victory by both sides in the dispute. The Dow Chemical Company points out that the skin cancers could have been caused by exposure to sunlight; the veterans' lawyers said the study showed that science was beginning to "catch up" with what the veterans already knew — that Agent Orange damaged their health.

The Ranch Hand study, however, is only one of more than 60 federal studies of the effect of dioxin being conducted at a cost of some \$100 million a year. Because the number of Ranch Hand personnel was small, the study was not expected to be sensitive to rare illnesses, a point made forcefully by a National Academy of Sciences review of the Ranch Hand protocol in 1980. Meanwhile, a major research programme, including a study of identical twins of whom one brother served in Vietnam, is under way within the Veterans Administration.

At congressional insistence, however, the Veterans Administration has had to transfer responsibility for a major epidemiological study of veterans to the Centers for Disease Control (CDC) in Atlanta. The Veterans Administration is distrusted by veterans and has been accused repeatedly of reacting slowly and insensitively to veterans' anxieties about their health. The \$70 million epidemiological study will be the biggest ever conducted by CDC. Some 30,000 veterans will receive a detailed health interview and another 10,000 will be given extensive two- or three-day physical examinations in what CDC hope will become the definitive analysis of the Agent Orange question. But the results will not start to emerge for at least seven years, too late to help the Brooklyn jurors in May. **Peter David**

Chinese nuclear industry

Advance on broad front

DISCUSSIONS on a possible \$20,000 million deal in nuclear technology were a major feature of President Reagan's visit to China last month. Coincidentally, the President's visit came shortly after the second national congress of the Chinese Nuclear Society, which reviewed the current state of Chinese nuclear technology and explored ways and means of "uniting and organizing" large numbers of scientists to meet the challenge of the nuclear age.

Addressing the inaugural session of the congress, Defence Minister Zhang Ai-Ping noted that although China has built a "fairly comprehensive" nuclear expertise over the past 20 years, and has started construction work on its first nuclear power station, at Taishan, the application of nuclear technology to the economy has been neglected. Satisfying the needs of "economic construction and improving people's standard of living" must, he said, be a priority for the nuclear industry, although it must also "ensure the satisfaction of military needs".

In fact, considerable progress has already been made on the applications of nuclear technology. More than 1,700 nuclear radiation instruments are now used in the textile, printing, paper-making, plastics and petrochemical industries. Between four and five thousand oil wells have been logged by radioactive methods, radiation sand-gauges have been installed in 12 hydrometric stations along the Yellow River to monitor sand content of the water and tracer techniques used for safety surveys of reservoirs.

Future plans include two more nuclear power stations (one in Zhejiang Province and one in the Shenzhen Special Economic Zone) and two 450-MW combined heat and power units for the Jinshang petrochemical works in Shanghai. Feasibility studies are in progress for urban central heating systems. According to Jiang Xinxiong, the Minister of the Nuclear Industry, China plans to be self-sufficient in nuclear fuel by the end of the century, while experimental work on fast breeder reactors and controlled thermonuclear fusion is "making progress".

The main problem, according to Defence Minister Zhang, is, however, safety, and, at the same time, popularization of knowledge about the peaceful use of nuclear energy in order to dispel "misgivings and apprehensions". The Chinese nuclear industry, Zhang said, is quite capable of handling fuel reprocessing, but should go on improving facilities and techniques in this field. Some experts believe that it could also undertake reprocessing on a commercial basis for other countries. **Vera Rich**

Australian technology

Jones and Hawke make hay

Canberra

THE restructuring of Australian industry in the face of rapid technological change has become a central policy theme of the year-old Labor Government, due in large measure to the advocacy of the Minister for Science and Technology, Mr Barry Jones. The Prime Minister, Mr R.J. (Bob) Hawke, has set up a committee of federal ministers to coordinate policies relating to the many deep structural changes necessary. As well as Mr Jones, the committee includes the Ministers for Trade, Education and Youth Affairs, Industrial Relations and Defence Support, under the chairmanship of Senator John Button, Minister for Industry and Commerce.

On 13 April, Mr Jones released a draft national technology strategy to stimulate Australia-wide discussion of structural problems. His paper, a summary of deliberations of the National Technology Conference last September, contains several proposals crucial to the transformation of Australian productivity. It is particularly concerned with the minuscule investment in research and development by private industrial companies. According to the figures made available, research and development in Australia in 1981–82 hit a low of 1 per cent of gross national product (GNP), with the business sector's contribution sinking to 0.2 per cent. In 1968–69, private research and development was 0.5 per cent of GNP in a national total of 1.3 per cent.

The paper now sets a target of 1.5 per cent of GNP overall for 1990, with the business share amounting to 0.5 per cent and rising to 1.0 per cent by 1995 to equal the government's projected contribution.

Much of the blame for Australia's dismal export performance in technology was attributed to the country's attitude to education as an investment. The Jones strategy now urges that the proportion of Australian children completing secondary school be increased from 30 per cent in 1982 to 40 per cent in 1985 and 50 per cent by 1995. Alarming, the number of tertiary students between 17 and 22 years old actually fell, as a proportion of the population of the same age, from 11.5 per cent in 1975 to 10.5 per cent in 1982. The strategy paper's recommended figures for 1985 and 1995 are 12 per cent and 20 per cent respectively.

Many of the recommendations in this paper are politically sensitive, including the proposal for a review of union attitudes to adult apprenticeships, the effect on training of minimum wages for juniors and the previously sacrosanct doctrine of "comparative wage justice" in which "equal work" attracts equal pay regardless of the industry or its capacity to pay. One per cent of the workforce should be re-trained each year, a portable super-

annuation scheme instituted to promote mobility between industry, universities and government, and industrial protection gradually reduced.

In a society arthritic with structural rigidity, with 50 per cent of the workforce unionized and a cadre of industrial managers not yet weaned from protectionism, the issues canvassed are strong meat. The prosecution of the policy implied will depend on Mr Hawke's skill at maintaining consensus.

An admirer of South-East Asian industrial development and a recent convert to economic planning in the style of the Japanese Ministry of International Trade and Industry (MITI), he claims that previous governments have not been interested in formulating coherent science and technology policies. **Jeffrey Sellar**

European pharmaceuticals

Free trade makes trouble

A BRITISH Medical Association (BMA) campaign aimed at preventing patients from being dispensed unlicensed drugs has suffered a temporary setback. The newly-formed Association of Pharmaceutical Importers claims that BMA's advice to physicians last week to stamp prescriptions "UK-licensed products only" contravenes the Treaty of Rome.

BMA's general medical services committee had become concerned by the growing number of reports of adverse reactions to imported drugs that are dispensed in the same ways as UK brands but which have not been licensed specifically for the UK market. Imported products bearing the same name as home lines may include differently coloured tab-

Radioactive atolls

Bikini Islanders sue United States

Washington

THE inhabitants of Bikini Island, which was used for 23 above-ground nuclear tests between 1946 and 1958, are suing the US Government in an attempt to force a clean-up of their homeland. The 167 residents of the island left at the request of the US military and have since been shifted several times to different nearby islands, living largely on food and supplies provided by the US Government.

A study commissioned by Congress reported last fall that a clean-up could be carried out at a cost of \$90–120 million.



The most feasible approach, according to the study team headed by Harvard professor emeritus Henry Kohn, would be the removal of a million cubic metres of contaminated topsoil. Without such drastic measures, the island would not be habitable for 100 years if local foods were to be consumed.

The urgency of the suit filed this week, according to the islanders' attorney

Jonathan Weisgall, is that the Compact of Free Association approved by the Marshall Islanders (of which Bikini is a part) and now pending before Congress would terminate all claims arising out of the nuclear testing, and would provide no funds for cleaning up Bikini. The Bikini Islanders would, however, receive \$75 million in compensation over the next 15 years on top of the \$23 million trust fund already set-aside by Congress. This fund is used to pay medical and other expenses and a monthly stipend to the 1,100 islanders and their descendants.

Eniwetok, an island near Bikini also used for nuclear testing in the 1950s, was cleaned up by the United States in the 1970s at a cost of \$105 million.

The Bikini atoll had been resettled after the Atomic Energy Commission (AEC) reported in 1967 that radiation had diminished to a safe level. In 1975, however, an initial ground radiological survey raised doubts about AEC's earlier conclusion; and a full survey in 1978, which included measurements of body-burdens of radiation in the 139 Bikini Islanders who had returned, found that they were receiving exposures of 0.27 to 1.18 rem per year, largely from eating locally-grown food that had taken up radioactive caesium-137 from the soil. The federal standard for the general public is 0.5 rem per year; the occupational limit is 5 rem per year. Those who had resettled were removed once again, and the island has since remained uninhabited.

As well as taking legal action, the islanders are appealing to Congress for action on a clean-up; the House Interior Committee has recommended an initial \$10 million authorization to begin the work.

Stephen Budiansky

lets and dosages, differently worded instructions (sometimes not in English) and may even be of different formulation or make-up. Its recommendation to physicians was intended to counter this trend.

BMA's advice is heartily endorsed by the Association of British Pharmaceutical Industry. British manufacturers estimate they lost £75 million last year to "parallel imports", bought from their overseas divisions and sold in Britain considerably below the standard British price. The price differentials are a consequence of the varied methods for regulating pharmaceutical companies in different countries. In Britain, overall profits are limited under the Pharmaceutical Prices Regulation Scheme, which specifies a target profit band for each company, based on capital employed, and also controls advertising expenditure. In other countries, prices or profits may be controlled individually for different products.

The European Court of Justice ruled in 1976 that parallel importing should be facilitated and that restrictions would be in breach of freedom of trade, but in 1980 an attempt by the commission to introduce a directive legitimizing parallel importing failed. The new pharmaceutical importers' association justifies its present imports by citing a British licences exemption order intended to apply "in circumstances in which small quantities of medicinal products are imported into the United

Kingdom for the treatment of particular patients".

Even though advertising of products imported under this order is illegal, the Department of Health has been unwilling to take action against infringements, fearing it would be dragged through the European Court. A new statutory instrument that will permit advertising of parallel imports and comply with the European Court's ruling while retaining some controls is now being finalized by the Department of Health and will be laid before Parliament in a few weeks' time.

A spokesman for the parallel importers accepted that British patients had in the past been dispensed products other than those their physicians intended, but said the association will encourage its members to "put their houses in order". Recently, some of its members were obliged to give an undertaking in the High Court to stop passing off as the UK version an imported Glaxo product with deliberately counterfeit packaging.

At issue now is whether a voluntary professional association such as BMA can make recommendations to its own members without contravening European law. BMA says it took full legal advice before making its recommendations to physicians and is confident of their legality. The question now seems likely to be answered in court, and BMA has halted its campaign until the issue is resolved to its satisfaction.

Tim Beardsley

China in space

Triumph and martyrdom

CHINA'S space planners last month released details of the setbacks and disasters suffered during the nine-year effort to put into synchronous orbit an "experimental" communications satellite. The satellite was successfully launched on 8 April, and inserted into its final orbit at 125°E on 16 April.

The admission on Chinese radio that a number of "scientific and technical workers" had "laid down their precious lives" during the development of the satellite was clearly intended as part of the current drive against "anti-intellectual" prejudice still persisting from the Cultural Revolution.

The broadcast centred on two major examples of heroism. One, a lone martyr, Ma Jungyan, had been working on the possible effects of solar radiation on a satellite in orbit. As this was during the "chaotic time of the Gang of Four", Ma was obliged to carry out simulated experiments at the atomic energy research institute without even the "minimum safety protection". He was, said the commentator, fully aware of the danger but "knowing that there was a tiger in the mountains, he still headed towards them". As a result, he developed a serious radiation-induced lung condition, but during

every remission continued to "work frantically".

The other incident was the explosion of a rocket on 28 January 1978, during tests on fuel flow. Seven of the research team, including the director of the test-pad, were seriously injured and several others suffered burns and ruptured eardrums. Nevertheless, at the urging of the injured director, a party meeting was held the same afternoon to determine the cause of the incident, and testing was back to normal three days later. A week after the incident, the director and "several other comrades" were back at work, still in bandages, and they returned to the hospital to complete their treatment only when the experiment was finished.

It is not clear whether there were any deaths in this incident; the commentator clearly implied, however, that there had been several fatalities on what he called the "battlefield" of the space programme, the programme of the rocketry division. Whether Ma Jungyan and his fellow martyrs will receive public recognition in the forthcoming exhibition on the Chinese space programme, which opens in Shanghai later this month, has not yet been revealed. But China seems bent on being open.

Vera Rich

Unesco

Looking for signs of change

FURTHER signs of the response of Unesco (the United Nations Educational, Scientific and Cultural Organization) to the discontent of some member states should become apparent next week, at the meeting of the organization's executive board arranged for 9-23 May. Those participating in the meeting expect that decisions about the budget for 1985, when the US notice of withdrawal will have expired, cannot be taken until the meeting of the executive board due in November. But next week's meeting should throw some light on Unesco's response to the British Government's for radical changes in the organization's way of working.

The agenda for next week's meeting concern's Unesco's science and technology sector only in uncontroversial ways — the executive board is expected to take on the nod the secretariat's proposals for the invitation lists to this November's meeting on information technology and that planned for next year on problems affecting Latin America and the Caribbean. Moreover, science and technology are relatively well protected in the secretariat's proposals for trimming \$10 million from the two-year budget for 1984-85. Indeed, the grant to the International Council of Scientific Unions has actually been increased.

Fireworks, if there are any, will rather attend the general items on next week's agenda, especially that requiring a first response to the British letter from Mr Timothy Raison, Minister of State at the Overseas Development Administration, sent to the director-general of Unesco in March. That letter asked that Unesco should reorder its priorities and improve the management of its affairs. At a later meeting in London, the director-general was told that continued British membership "could not be justified" unless there were signs of change at Unesco.

By all accounts, few expect that there will be much that is tangible to report from this first meeting of the executive board since the letter was sent, although the British will at least be looking for some mechanism by which their proposals can be studied seriously. But the responses of both the Unesco secretariat and the delegates of other member states are likely also to influence the spirit in which the British Government carries out its promised — or threatened — review of the benefits of membership.

Mr Ray Beverton, who succeeded Sir John Kendrew on 1 April as chairman of the science and technology subcommittee of the British National Committee on Unesco, said earlier this week that his group had not yet begun work in earnest on the task, but that a meeting had been arranged for May. □

Animal welfare

UK compromise and coercion

THE British Toxicology Society has thrown its professional weight behind the campaign by British animal welfare groups to end the LD₅₀ test of acute toxicity. A special report by a working group of the society concludes that accurately determined LD₅₀ values are rarely justified, and proposes an alternative test which it considers more humane while still providing essential safety data for product labelling and classification.

The LD₅₀ test has long been a main target of animal welfare campaigners. New guidelines published in 1981 by the Organization for Economic Cooperation and Development (OECD) lowered the number of animals needed for the test, which establishes the quantity of a substance needed to kill 50 per cent of a group of test animals. Even with the new guidelines, however, 30 animals would typically be used for each LD₅₀ determination.

The alternative proposed by the British Toxicology Society classifies substances into broad bands of toxicity based on their observed effects on animals at pre-set dosages. If survival at one level is more than 90 per cent, with no evident sign of toxicity, dosage is increased by a factor of 10; if it is less than 90 per cent, dosage is decreased by the same factor. This procedure continues until the pre-set level is found that causes evident toxicity but allows more than 90 per cent survival, which specifies the classification. With 10 animals per group and 3 classes of toxicity (as in the current EEC chemical regulations), on average between 10 and 20 animals would be used.

The Home Office in London is working on new proposals for animal welfare legislation, and is keenly aware of public pressure to reduce the number of animals used in routine testing. Mr David Mellor, Under-Secretary of State at the Home Office, is known to be sympathetic to the view that the continued widespread use of LD₅₀ should not be necessary. The Toxicology Society hopes its acute toxicity test procedure will gain peer acceptance and become internationally recognized.

The claimed advantages, apart from compatibility with existing European systems, include the fact that (unlike LD₅₀) signs of toxicity are included in the assessment, while severely affected animals can be humanely killed without affecting the outcome of the study. The new test is also likely to be less variable between laboratories than LD₅₀. The long-term aim of the society is to force a European or a UK initiative in OECD aimed at establishing new guidelines. An existing EEC directive on dangerous substances that seems to imply use of the LD₅₀ test would probably need amending.

Meanwhile, the Home Office is still undecided on how to modify the controversial

"pain clause" in the draft proposals on animal experiments that it published last year. It is being pressed by the British Veterinary Association and others to extend the existing ban on experiments that cause severe and enduring pain to those causing severe pain or distress. The association, together with the Fund for the Replacement of Animals in Medical Experiments (FRAME), is also pressing for a linkage between the amount of pain or distress that may be allowed in an experiment and the potential benefits. Home Office officials are now thought ready to accept the principle of such a linkage.

Civil disobedience and violent protest against animal experiments are likely to be

stepped up over the coming months in Britain. A coalition of hardline antivivisection organizations linked under the banner of "Mobilisation for Laboratory Animals" is planning a week of protests that will culminate in a national march on 12 May. The demands include a ban on the LD₅₀ test and the Draize eye test, as well as cosmetics testing and all behavioural, psychological and military experiments. Last weekend, a security guard was injured when protesters broke into testing laboratories owned by Imperial Chemical Industries Ltd. Some parties to the "mobilisation" coalition condone, if not actually organize, protests that entail physical violence, and more violent incidents seem likely. Later in the year, according to a spokesman, a vigil will be held outside every animal laboratory in Britain.

Tim Beardsley

Israeli-Egyptian relations

Cooperation in the doldrums

Rehovot

A LEADING Israeli physicist recently gave an "unofficial lecture" at a major Egyptian university. For political reasons, there was no advance notice of the lecture, organized by an Egyptian colleague whom the Israeli had met at a US conference. Word got around, however, and by the time the Israeli began to speak, the lecture room was crowded with young Egyptian researchers, most of whom stayed behind afterwards to discuss physics and to express the hope that full above-board links between Israeli and Egyptian scientists would soon be possible.

Although Israel and Egypt have been at peace for five years, there is at present only one place where scientists and scholars from the two nations can meet officially, the Israeli Academic Centre in Cairo headed by Professor Shimon Shamir of Tel Aviv University. The centre, sponsored principally by the Israel Academy of Sciences and Humanities, functions like the cultural centres of other nations, serving Israeli academics in Egypt for scholarly research, presenting lectures by these academics and giving Egyptian scholars access to Israeli research papers and books.

Such activities would be quite straightforward were it not for the fact that many in Egypt now regard all contact with Israel, no matter how innocent, as suspect.

Since the centre opened in May 1982, most of the lectures given there have been devoted to subjects of clear mutual interest. For example, Professor Sasson Somekh, an Israeli expert on Arabic literature and language, dwelt upon the fact that Hebrew and Arabic originate from the same linguistic family — and then dealt with problems of modernization in the same spirit. Similarly, Israeli musicologist Amnon Shiloah examined the influence of Arab music on Jewish music while Hebrew

University Professor Hava Lazarus-Yafeh discussed the relationship between Halkha (Jewish religious law) and Sharia (Moslem religious law).

Sometimes, Israeli lecturers have reported on research carried out in Egypt itself. Dr George Kanazi, a senior lecturer in Arabic literature at Haifa University, told his Cairo audience about mediaeval Arabic manuscripts on the art of wine-drinking. Kanazi, himself a Christian Arab born in Nazareth, pointed out that even after the advent of Islam, which prohibits intoxicating beverages, laxity in the interpretation of Islamic law had made wine-drinking "a luxury that was sought and enjoyed".

Many of the Israeli researchers visiting the academic centre are interested in Islam and many of the Egyptians are similarly interested in Judaism.

Egyptian doctoral candidates have received information, for example, on "Elijah the prophet in Jewish and Moslem folklore" and "the author Aharon Megged as a representative of Israel's 1948 generation". Other PhD candidates have sought and obtained material for studies comparing Egyptian and Israeli approaches to education, social welfare and architecture.

The reactions of Israelis to these interactions, and to Egypt generally, are variable. Shamir says that "Israelis who come to Egypt looking for fanaticism, political rigidity and uncompromising hostility towards their country find these things; those who come looking for open-minded attitudes, warmth and a desire for peace find them also."

Shamir regrets that it is impossible to reach agreement just now for scientific cooperation between Israeli and Egyptian research centres but welcomes "the beginnings of a cultural interchange" at this centre.

Nechemia Meyers

Amersham International

Academic symbiosis flourishes

To sponsor a fellowship at the University of Cambridge, in a department (biochemistry) run by a knight (Sir Hans Kornberg) who is also master of a Cambridge college (Christ's), as Amersham International is about to do, may seem slightly odd for a company that thinks of itself as an adventurous creature newly emerged from the confining chrysalis of public ownership. But the Amersham Fellowship is a recognition by the company of the extent to which its growth, diversification and profit depend upon the progress (and demands) of academic research.

Dr J.C. Maynard, chief executive of the research products division — one of three into which the company is now organized — estimates that he has about 120 collaborations with academics, including sponsorship of several projects. That is largely why the company was able to market 130 new research products last year, many of them the result of an opportunist approach and many of them unrecognizable as products of what was, until 1981, the Radiochemical Centre.

The change of name followed a change of managing director and preceded the sale in 1982 of shares in the company by the British Government. (That move is reckoned to have cost the taxpayer dearly because of a gross underestimate of investor demand for the shares, which more than doubled in price after the sale.)

One striking change of business strategy has been that Amersham is taking a leaf out of the British dairy business's book. Just as the system of daily delivery of milk to British households is now being exploited to offer anything from meat to bread, so

Amersham has decided to exploit its system for the speedy delivery of labile radiochemicals to market other products. The main thrust has been in the direction of molecular biologists. Since they all take deliveries of radiochemicals, why not offer them restriction enzymes as well?

Amersham seems to have made a considerable success out of just that, obtaining the enzymes from the Japanese market leader, Takara Shuzo. Perhaps five per cent of the sales of research products, which accounted for about a third of the company's £78 million turnover in 1983, is accounted for by restriction enzymes. But, as marketing manager Dr Brian Ellis is quick to say, Amersham faces much more competition in the sale of enzymes than in radiochemicals. Like all enzyme companies, Amersham would dearly like to have some unique enzymes on its list and would also like to offer reverse transcriptase, an essential enzyme for almost all molecular biologists.

Other burgeoning sales to molecular biologists come from reagents for the cloning, packaging and sequencing of DNA. Most of the reagents are not radioactive, many are offered in convenience kits. (There is even an anti-DNA kit, not so much an anti-research product as an aid to diagnosing the presence of antibodies to DNA in clinical blood samples.) Like many other companies, Amersham is also looking hard at the possibility of supplying non-radioactive alternatives to the current DNA probes but so far none is of sufficient sensitivity, says product manager Mahomed Jassat.

More in the traditional line of the company, but typifying its new approach,

Taxing problems

MANY readers of *Nature* who have been alarmed to learn that foreign visitors earning income in the United Kingdom will in future be taxed like residents (*Nature* 26 April, p.762) have nothing to fear. The proposed changes in tax relief for non-domiciled employees working in the United Kingdom for non-resident employers, now in committee stage in the British Parliament, do not affect those working as teachers or professors at a recognized educational establishment, or those who are undertaking research that is for the benefit of the general public.

People in these categories will continue to enjoy total freedom from taxation in the United Kingdom, provided that the visit is for less than two years. Under double taxation relief agreements, this exemption is reciprocal and so would apply also to British academics visiting foreign countries to teach or carry out research.

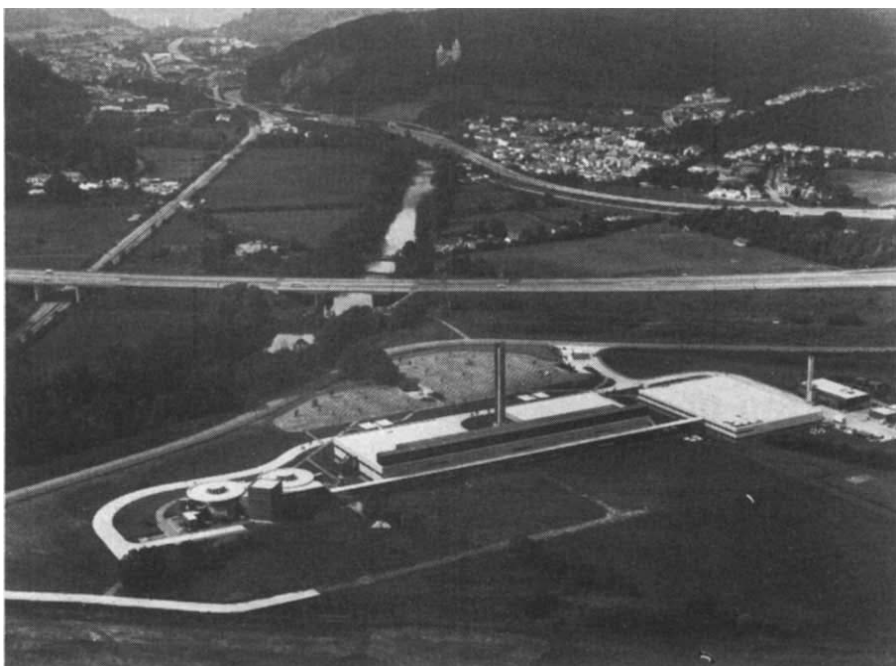
Those unfortunates who are not teaching, or whose research is for the benefit of private individuals or companies, will not in future benefit from the special reduction for short-term visitors to the United Kingdom, currently calculated at a deduction of 50 per cent on earnings. From 1987 the relief will be reduced to 25 per cent and from 1989 it will be withdrawn entirely. □

are radioisotopically labelled ligands for receptors on the surfaces of cells. "It's a flavour of the month business", says Ellis. The trick is to be very quick off the mark to produce new ligands after their discovery and before they are displaced by something better. It can take Amersham as little as three months to launch a new product. Some may sell for not much longer than that, and to the tune of only a few thousand pounds, but still be worthwhile.

Financially as well as physically, Amersham has been growing since it went public in 1982. The half-year figures for 1983 show sales and profits more than 20 per cent up on 1982. More than 80 per cent of sales are overseas, business is booming in Japan and the company feels it is gaining on its arch-rival, New England Nuclear, in the United States. In physical terms, the staff of Amersham now tops 2,000, the administrative staff is to move into offices being built close to the Amersham site and research staff will soon start to occupy what was the Pollards Wood Research Station of the Institute of Cancer Research, a few miles away.

That move is part of the two-year plan under which expenditure on research and development will increase from 8 per cent of sales to 10 per cent. As long as price resistance does not hit sales, Amersham will soon be in better shape than ever to take advantage of the many ideas that seem to be flowing from its contacts in academic life.

Peter Newmark



Signs of growth — Amersham's new facilities at Cardiff.

Labs worth visit

SIR — Your cheap sneer (*Nature* 15 March, p.24) that “many once adventurous laboratories have become places where ageing investigators brood on their pension rights” perhaps reflects the fact that your reporters are no longer visiting these laboratories. If they did, they would recognize the exciting and high calibre research which is being conducted against a background of continuing cuts in both staff and equipment. They might also recognize the high regard with which these laboratories are held by overseas scientists if we are to judge by the number of requests received from scientists in developed and developing nations alike to visit and work in these laboratories.

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Nuclear abstracts

SIR — M. A. Bray (*Nature* 307, 206; 1984) had expressed concern about the large number of abstracts appearing in databases. J. R. Metcalfe (*Nature* 308, 222; 1984) questioned whether conference abstracts should even be included in databases.

For the nuclear physics bibliographic databases maintained at the US National Nuclear Data Center (NNDC), secondary references such as meeting or conference abstracts and dissertation abstracts are routinely coded. In the bibliography to neutron-induced reactions (CINDA system), all references to a given research work (either experimental or theoretical) are blocked together in the database. Abstracts are entered with a “no book flag” so that they never appear in the semi-annual cumulative publication, unless the abstract constitutes the only reference in that block. However, all entries can be obtained in a computer retrieval. The same was true for the charged particle induced reaction bibliography (CPBIB) system.

For the nuclear structure bibliography (Nuclear Structure References system) which is published thrice annually, there is no blocking. However, the secondary references appear in a section separate from the primary journal listings. In support of Metcalfe's arguments in favour of inclusion, the interval between an initial abstract and a published paper can be from one to five years. This time factor is significant (1) to alert a potential researcher that another group is already working on the same problem which could possibly avoid duplication and wasted research funds and (2) to direct a potential user to the researcher for more details on the work prior to publication.

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They are not alone

SIR — Is the individual research scientist becoming an endangered species, as more and more disappear into the relative obscurity of “research teams”? An investigation of the pages of *Nature* suggests that this is indeed the case.

Since 1979, the number of “Letters to *Nature*” published has been in the region of 1,200 per year. However, throughout this period, there has been a gradual annual reduction in the number of single — and double — author papers coupled with an overall decrease in manuscripts with three named authors. This marked decline has been accompanied by a major increase in the number of multi-author papers.

A breakdown of the 1979 and 1982 quota of Letters to *Nature* clearly illustrates the change of distribution. During the four years, the decrease in letters with three authors or less was reflected by the steady rise in the average number of authors per letter, the 1982 figure standing at a mean of

Numbers of Letters to *Nature* with 1 to > 9 authors in 1979 and 1982.

	1	2	3	4	5	6	7	8	9+
1979	198	448	307	161	65	34	13	9	11
1982	134	360	265	170	83	67	28	17	12
Mean change per year (expressed as %)	-9	-7	-2	+5	+13	+35	+42	+39	+7

Homer's vino

SIR — Will someone in the Mediterranean please confirm or refute Wright and Cattley's chemical hypothesis about Homer's “wine-dark sea” (*Nature* 303, 568; 1983). I tried with French wine using Copenhagen water that is alkaline enough to turn red cabbage blue, but all I got was a tasteless red fluid. Perhaps “wine dark” refers to the lack of turbidity in wine. Coastal and inland waters are usually turbid whereas the clarity of deep Mediterranean waters off rocky shores could be compared to the clarity of wine. If so, “wine clear sea” would be a better translation and close to the Dutch/Afrikaans (?) *wijnkleurige zee* quoted by Macnamara (*Nature* 307, 590; 1984).

The use of οἶνον ποτῶν as a fixed Homeric metaphor without necessary reference to colour has an analogue in modern English. What is the skin colour of most of the white men you have seen recently?

ROBERT B. DEAN

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Exciting news

SIR — Your reviewer (*Nature* 307, 765; 1984) suggests that some results may “titivate”. It is hard to see how they could spruce up either themselves or your readers, although the results might well agreeably excite (titillate) at least the latter.

R. P. BOAS

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3.17 authors per paper which represents a 14 per cent increase overall.

The observed shift towards multiple authorship in *Nature*, illustrated by the presence in 1980 of a 2½ page article with 27 named authors, poses the question: has scientific research become specialized to such an extent that individuals must now pool their expertise in order to successfully carry out a research project?

The most likely cause for the conspicuous movement away from single-authorship is, perhaps the “publish or perish” syndrome. Drastic funding cut-backs in the private and public sectors, worldwide recession and the threat of unemployment have all intensified the pressure on researchers to protect their positions. Being named on as many publications as possible, no matter how remote the connection with the original research work, is one way of proving one's competence.

ANDREW J. CRUMP
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Only correct

SIR — Two errors are becoming commoner in scientific writings.

(1) The word “only” is being shifted forward, away from the word it modifies. Note the differences among the following: “Only I saw the white shark” (no-one else was on deck). “I only saw the white shark” (I didn't actually catch it). And “I saw only the white shark” (there were no grey ones around).

(2) Within the past few years the word “within” has been supplanting the simpler “in”, usually without cause. Rats live within cages, particles sediment within a limited space, trees within a given area grow and die. In such contexts, what is wrong with using “in”?

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The real thing

SIR — Your correspondence columns have recently contained proposals for various “opposites” to placebo. The German-speaking world has used *Verum*, “the true thing”, happily for years as an antonym to placebo. I managed to use it in an English language journal (*J.R. statis. Soc. A* 146, 386; 1983), although the editors insisted on printing it in italics.

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Benefits of measuring time

Further data from the eccentric binary pulsar reaffirm the value of observations extended over time. The reality of gravitational waves is confirmed, the masses of the stars remain a puzzle.

THE old adage that if you must make measurements, you had better use a clock for then you will have time on your side, has been verified again. Almost exactly five years ago, Professor J.H. Taylor, the radioastronomer then at the University of Massachusetts at Amherst, described the first measurements of what is affectionately known as his pulsar (*Nature* 277, 437; 1979) that were accurate enough to constitute a proof that gravitational radiation is real. In fact, the object that Taylor has been observing closely for the best part of a decade is not an isolated pulsar, but a binary system — a neutron star and a companion star of roughly equal mass.

The good luck is that the two objects are tightly bound — the orbital period is 7 hours 45 minutes — and that the orbit is also markedly elliptical. And the consequence of that is that the precession of the orbit resulting from non-Newtonian gravitational forces is rapid — more than 4 degrees a year, compared with a mere 0.43 seconds of arc a year for the precessing of the perihelion of the planet Mercury in its orbit about the Sun. Gravitational waves enter because the orbital period (not in this connection to be confused with the rotational period of the pulsar of 0.059 seconds) is decreasing, which implies that the system is losing energy in a way that can be explained only by gravitational radiation.

Taylor, now at Princeton University, has now, with J.M. Weisberg of the same laboratory, brought the story of the pulsar PSR 1513-16 up to date (Weisberg and Taylor, *Phys. Rev. Lett.* 52, 1348; 1984). Observations with the 300-m Arecibo dish in Puerto Rico have been accumulating for the whole of a decade. Altogether, there are more than 3,300 separate measurements, each consisting of the integration of the 5,000 or so pulsar wave forms received in a 5 minute interval. The authors say that their binary system is "uniquely suitable" for testing the prediction of general relativity that the members of a binary system should "gradually spiral together as the system loses energy in the form of gravitational radiation", but many of their readers will be more impressed by the astonishing precision with which the orbit of the binary system can now be described.

Inevitably, the orbital period is the most accurately determined of all the quantities involved. The standard error of the period, in round numbers 27,907 seconds, is a mere 20 microseconds, comparable with the

standard error of a single measurement of the time of arrival of the individual pulses in one of the elementary observational periods of 5 minutes. But with a terrestrial atomic clock accurate to 1 microsecond, should it not have been possible to do even better after 10 years of observation? With more than 1,000 orbits every year, 10 years of observations should after all give a periodic time accurate to within the standard error of the clock divided by 10,000, say a nanosecond or so. And are not the rotational periods of many pulsars known to an accuracy of better than 1 picosecond?

The snag is that a system is much more complicated than an ordinary bare pulsar. With both kinds of objects, of course, it is necessary to correct the times at which pulses are expected to arrive for the physical motion of the Earth in its orbit around the Sun (which can make a difference of more than 2,000 seconds for an object in the plane of the ecliptic). For a binary system such as Taylor's, it is necessary to extract from what is essentially a sequence of time-marks all the characteristics of the pulsar orbit. One practical difficulty with Taylor's pulsar is that its signal is weak, though reproducible, so that the usual trade-off between receiver bandwidth and time precision arises.

With the passage of five years, however, the precision with which quantities such as the eccentricity of the orbit (numerically, 0.617 and now known to 3 parts in a million) can be known has been improved by a factor of at least two. The most striking improvement has come in the estimate of the projection of the semi-major axis of the elliptical orbit on the line of sight (a factor of five), the rate at which the orbital period is decreasing (a factor of seven) and, most important, the rate of precession of the orbit (a factor of six). A decade from now, this distant orbit may be as well known as, say, the orbits of some of the satellites of other planets in the Solar System.

Conceivably, the precision of some of these quantities may be dramatically improved by a technique Dr A.G. Lyne of the Nuffield Radio Astronomy Laboratories of the University of Manchester at Jodrell Bank has been exploiting in the study of another binary system one of whose members is also a pulsar. By all accounts, it is possible to exploit the irregular pattern of ionization in the general direction of a pulsar which is part of a binary system to

infer the position of the pulsar in its orbit from the scintillation of the pulsating radio signal. For practical purposes, the ionization pattern in the intervening space functions as a kind of scale against which the position of a pulsating star can be pinned down. No doubt Taylor will be anxious to see whether the space intervening between here and PSR1513 + 16 allows this to be done.

Even as things are, however, Weisberg and Taylor have a good deal to say. In 1982, Taylor and Weisberg (*Astrophys. J.* 253, 908; 1982) were saying that the data accumulated by then ruled out all gravitational theories except general relativity and the Brans-Dicke theory. Now they say that only Einstein's theory will square with the observations.

Weisberg and Taylor also say that their data now reveal a delay in the signal from the pulsar in those parts of the orbit from which the line of sight passes through the gravitational field of the companion star. This is the phenomenon now frequently observed when the line of sight to a pulsar grazes the limb of the Sun. Weisberg and Taylor say that their data constitute the first observation of this effect outside the Solar System.

The other striking and newish development is that the masses of the two stars have now been pinned down much more precisely than has previously been possible. The essential difficulty is familiar — that the masses of celestial objects whose gravitational interactions are those of point masses cannot be derived from Newtonian mechanics. But for the binary pulsar, the relativistic corrections, the advance of the periastron for example, are necessarily the least certain. But these are precisely the properties of the system whose precision has been most strikingly improved in the past five years. And the outcome makes more remarkable what has seemed from the start a strange coincidence — the masses of the two stars are indistinguishable both from each other and from the Chandrasekhar limit for collapse to a neutron star. So is it just possible that each of the stars is a neutron star, but that only one of them is a pulsar? It may be possible to learn something about the nature of the companion star if the measurements become precise enough for mutual tides to show up, but on the face of things not even the passage of time will allow this intriguing question to be answered.

John Maddox

Acquired immune deficiency syndrome

Retroviruses linked with AIDS

from Robin Weiss

THE publication of four papers¹⁻⁴ in the 4 May issue of *Science* by R. C. Gallo and colleagues on a link between acquired immune deficiency syndrome (AIDS) and a retrovirus related to human T-cell leukaemia virus (HTLV) was preceded by much comment which has generated considerable confusion. The main causes of this sorry state of affairs are the intense interest in, and fear of, AIDS and the decision that the US Secretary of State for Health should herself announce 'the identification of the cause of AIDS' and hence the 'means of prevention'. Moreover, rivalry and lack of cooperation between different US governmental agencies — the Center for Disease Control, the National Institute of Allergy and Infectious Diseases and the National Cancer Institute (NCI) — led to uncoordinated press statements. These have exacerbated the already contentious matter of deciding precedence between L. Montagnier's group at the Institut Pasteur in France, which published first^{5,6} but with skimpy data, and Gallo's group at NCI, which delayed submission until a thorough characterization of their virus and repeated isolations from different patients had been accomplished. Furthermore, the two groups are using three different names for what I believe will turn out to be the same virus.

Why look for a virus?

There no longer seems to be any doubt that AIDS is caused by an infectious agent. The view that it might instead result from repeated exposure to different human antigens, while plausible for homosexuals with multiple sexual partners and for haemophiliacs receiving factor VIII pooled from thousands of blood donors, cannot apply to AIDS developing after a single blood transfusion or in infants of affected mothers. What kind of infectious agent, then, might give rise to this disease? Again, the discovery of the transmission of AIDS by blood or by semi-purified blood products helped narrow the field because it is unlikely that a fungus or a bacterium (perhaps secreting an immunosuppressive toxin comparable to cyclosporin A) would be transmitted by such routes. In any case, no sooner had AIDS been categorized as a new, epidemic and usually fatal syndrome in 1981, than the search for its causative agent was directed mainly towards viruses.

AIDS patients, and those with extended lymphadenopathy syndrome (ELAS) which may be either a milder form or early phase of AIDS, are infected with many viruses. Cytomegalovirus, Epstein-Barr virus and hepatitis B virus are examples of ubiquitous persistent infections in the pop-

ulation at risk as well as many other individuals. They may appear in more virulent form in AIDS and ELAS patients but that is probably a result rather than a cause of the disease. In turn, they may play a part in exacerbating immune deficiency once it sets in, but these well known widespread viruses did not seem likely to be the initiating agent of AIDS. Instead the pointers were towards a new virus, or a new satellite agent which might increase the pathogenicity of a common virus, just as the delta agent causes hepatitis B to be more severe.

Montagnier's viruses (LAV and IDAV)

Last May, Montagnier and colleagues reported the transient production of cell-free reverse transcriptase activity and virus particles when leukocytes from an ELAS patient were co-cultivated with fresh T cells from normal subjects⁵. The virus showed no cross-reaction with the core antigens of HTLV-1, the virus that is frequently associated with human T-cell leukaemia, though there was slight cross-reaction with antigens on the membranes of HTLV-1-infected cells. Montagnier named the virus lymphadenopathy virus one (LAV-1). Scepticism of the French group's observations grew because other laboratories had difficulty in confirming them and because of a widely held opinion among electron microscopists that the particles depicted in the paper resembled an arenavirus more than a retrovirus.

At the Cold Spring Harbor Meeting on HTLVs last September, Montagnier presented further data, including electron micrographs of particles that resembled D-type retroviruses with truncated (cylindrical) cores and long stalks where budding of the virus from the plasma membrane of infected cells was incomplete⁷. The major core antigen, p25, of LAV-1 was found to be antigenically related to equine infectious anaemia virus (EIAV), a little-studied retrovirus that causes haemolytic anaemia and fever in horses. In its ultrastructure, EIAV is similar to LAV-1 as seen in the more recent electron micrographs^{7,8}. At Cold Spring Harbor, Montagnier also described further isolates of retroviruses from two AIDS patients⁷, one of them a haemophiliac. The virus from the haemophiliac and a virus isolated from his brother, who is also a haemophiliac but does not have AIDS, have recently been described in *Lancet*⁶ and have been named immune deficiency associated viruses (IDAVs). IDAV is indistinguishable from LAV.

These viruses are promising candidates as aetiological agents of AIDS and ELAS

because 60–75 per cent of AIDS patients have serum antibodies that react with the p25 antigen of LAV-1, and because they are cytotoxic *in vitro* to the OKT4⁺ sub-group of T cells which contains the majority of helper T cells. To date, LAV-1, IDAV-1 and IDAV-2 have not been thoroughly characterized biochemically, and permanent cell lines producing the viruses are not yet available, although nearly a year has passed since the first report of LAV appeared in print⁵.

Gallo's virus (HTLV-3)

The virus now described by Gallo *et al.* has been designated HTLV-3 because it is considered to be a new member of the HTLV family (now called lymphotropic, instead of leukaemia, viruses). Some 50 HTLV-3 isolates have been obtained from different AIDS and ELAS patients with the ingenious use of a T-cell leukaemia line (HT) that is highly permissive to HTLV-3 replication¹. The infection of HT by HTLV-3 is easily detected by the formation of syncytia similar to those we described for HTLV-1 and HTLV-2 (ref. 9). The continuous production of high titres of HTLV-3 in HT allowed Gallo's group to characterize the viral proteins and prepare antibodies to them. Schüpback *et al.*³ report a cross-reaction of HTLV-3 p24 with other HTLV strains, particularly HTLV-2. Genome analysis, yet to be published¹⁰, indicates that HTLV-3 is significantly related to HTLV-1 and HTLV-2.

More than 80 per cent of ELAS and AIDS patients have serum antibodies that are related to specific HTLV-3 antigens, especially the envelope glycoprotein, gp41. A relatively high proportion of a small sample of homosexuals and drug addicts without AIDS also have HTLV-3 antibodies.

Are HTLV-3 and LAV/IDAV the same virus?

The NCI group place their AIDS virus firmly in the HTLV family whereas the French group have emphasized that their virus has antigens that cross-react with EIAV and that it lacks relationship to HTLV-1. Nonetheless, in my view the similarities between HTLV-3 and LAV are more remarkable than the discrepancies. Disregarding the original electron micrographs of Barré-Sinoussi *et al.*⁵, the ultrastructure of both viruses is very similar (compare Fig. 2 of Vilmer *et al.*⁶ with Fig. 1 of Gallo *et al.*²). While HTLV-1 and HTLV-2 endow OKT4⁺ cells with indefinite growth potential, neither HTLV-3 nor LAV has this capacity for 'immortalization'. Both HTLV-3 and LAV can be passaged as free virus, whereas HTLV-1 is much less stable and unlikely to survive the process of factor VIII preparation — although we have reported cell-free transmission of HTLV-1 (ref. 11).

The apparent differences in immunological cross-reactions could be explained by the different tests and reagents used by

the two groups, particularly as HTLV-3 is more distant from HTLV-1 than HTLV-2 and most AIDS and ELAS patients lack antibodies recognizing p24 of HTLV-1. Given the relationship claimed between LAV and ELAV it will be interesting to ascertain whether the latter belongs to the HTLV family (which includes bovine leukaemia virus). The relationship of HTLV-3 and LAV could be rapidly resolved by an exchange of reagents between the two laboratories.

A serological test devised in Essex's laboratory¹² (the 'HTLV-MA' assay) appears to correlate closely with AIDS and ELAS in homosexuals, haemophiliacs and other recipients of blood transfusions¹²⁻¹⁴. The HTLV-MA test is an immunofluorescence assay of the binding of serum to the surface of HTLV-1-infected cells, in particular to the gp61 envelope precursor of HTLV-1 that has been defined by Hattori *et al.*¹⁵. I have been frankly dubious about HTLV-MA as a specific test of HTLV-1 antibodies because of its lack of correlation with our more specific env antigen assays^{9,16} and because faint fluorescence at 1 in 4 dilutions of serum were counted as positive. It now seems much more likely that the HTLV-MA weakly cross-reacts with HTLV-3 or LAV. No doubt Essex *et al.* will re-analyse their sera using a more appropriate antigen. Indeed, as mentioned earlier, a weak cross-reaction by live-cell immunofluorescence between sera of LAV-positive patients and HTLV-1-infected cells has been observed⁵.

Immunodeficiency caused by other retroviruses

One of the reasons for suspecting that a retrovirus might be the cause of AIDS is that several animal viruses (including some strains of avian and feline leukaemia viruses) are known to cause various kinds of immunodeficiency, anaemia and aplasia. Indeed, it has not been lost on opportunistic retrovirologists that the US AIDS budget is a lucrative source of research funds. For years, the immunodeficiencies caused by some retroviruses were regarded as a nuisance because infected animals died of 'laboratory' infections before they could develop leukaemia. The focus of pathogenesis has curiously changed in recent months with an eruption of vintage viruses carrying new appellations such as FAIDS (feline AIDS) and SAIDS (simian AIDS).

The prototype D-type retrovirus, the Mason-Pfizer monkey virus (MPMV), was first isolated from a rhesus monkey in 1970 (ref. 17). Much effort was expended in attempts to demonstrate oncogenic properties of MPMV but as Fine and Schochetman's excellent 1978 review¹⁸ relates, the only pathogenic effect of MPMV was runting and immunodeficiency. Recently, outbreaks of simian 'AIDS' in the New England and California regional primate centres have been closely associated with a new strain of D-type virus

related to MPMV^{19,20}; experimental inoculation of this virus into young monkeys induces the disease²⁰. Simian AIDS differs from human AIDS in affecting a much wider spectrum of blood cells than OKT4⁺ T cells; nonetheless the identification of a retrovirus with this disease is of considerable interest. The human AIDS viruses are not antigenically related to MPMV.

HTLV-1 in AIDS/ELAS patients

HTLV-1, until recently known as ATL in Japan²¹, is most closely associated with adult T-cell leukaemia-lymphoma²², a malignancy of OKT4⁺ T cells. This clearly influenced Gallo's search for a virus in AIDS patients as it seemed reasonable to surmise from a knowledge of animal retroviruses that the same virus that initiated clonal malignant transformation in one situation, or a variant, might destroy the same target cell population in another situation. Another hint came from the fact that opportunistic infections, as characterize AIDS, frequently accompany the appearance of HTLV-1-induced malignancy.

Last year, Gallo's laboratory reported the isolation of HTLV-1 and proviral DNA from homosexual AIDS and ELAS patients^{23,24}, and the same virus has been identified in Haitian infants with AIDS²⁵. Using serological tests which do not cross-react with HTLV-3/LAV we have also found that about 5 per cent of ELAS patients in London have antibodies specific to HTLV-1 (ref. 26), a far higher incidence than in healthy homosexuals and random blood donors. The significance of HTLV-1 infection in subjects at risk of AIDS is not clear, but from its low prevalence it seems more likely to be a blood-borne infection reflecting the life style of the patients than an alternative aetiological agent of AIDS.

Prospects for controlling AIDS

If HTLV-3/LAV really is the cause of AIDS, and I find the evidence convincing, then several important developments should occur quickly. First, we need to

know what proportion of patients at risk actually have evidence of infection. The preliminary report⁴ that a substantial proportion of clinically normal homosexuals have HTLV-3 antibodies is worrying, if it is a forewarning of future illness; it is clear, however, that cofactors play an essential part in precipitating AIDS. Second, reliable ELISA screening tests for blood banks are urgently needed, and might have to be used as routinely as tests for hepatitis B virus. Third, there is the prospect of developing prophylactic immunization and perhaps intervention for infected individuals too. As Groopman pointed out in these columns last week²⁷, the economic and logistical impact of the identification of the cause of AIDS is formidable, and the seropositive individuals will face severe psychological and behavioural problems.

I am most grateful to R. C. Gallo and L. Montagnier for permission to utilize information in papers in press for this article. □

1. Popovic, M. *et al. Science* **224**, 497 (1984).
2. Gallo, R. C. *et al. Science* **224**, 500 (1984).
3. Schüpbach, J. *et al. Science* **224**, 503 (1984).
4. Sarngadharan, M. G. *et al. Science* **224**, 506 (1984).
5. Barré-Sinoussi, F. *et al. Science* **220**, 868 (1983).
6. Vilmer, E. *et al. Lancet* **i**, 753 (1983).
7. Montagnier, L. *et al. in Human T Cell Leukaemia Virus* (eds Gallo, R. C., Essex, M. & Gross, L.) (Cold Spring Harbor Laboratory, New York, in the press).
8. Montagnier, L. *et al. Ann. Virol.* (in the press).
9. Nagy, K. *et al. Int. J. Cancer* **32**, 321 (1983).
10. Wong-Staal, F. Personal communication.
11. Clapham, P. *et al. Science* **222**, 1125 (1983).
12. Essex, M. *et al. Science* **220**, 859 (1983).
13. Essex, M. *et al. Science* **221**, 1061 (1983).
14. Jaffe, H. W. *et al. Science* **223**, 1309 (1984).
15. Hattori, S. *et al. Gann* **74**, 790 (1983).
16. Clapham, P. *et al. Proc. natn. Acad. Sci. U.S.A.* **81**, 3083 (1984).
17. Chopra, H. C. & Mason, M. M. *Cancer Res.* **30**, 2081 (1970).
18. Fine, D. & Schochetman, G. *Cancer Res.* **38**, 3123 (1978).
19. Daniel, M. D. *et al. Science* **223**, 602 (1984).
20. Marx, P. A. *et al. Science* **223**, 1083 (1984).
21. Watanabe, T. *et al. Science* **222**, 1178 (1983).
22. Hanaoka, M. *et al. (eds) Gann Monogr.*, **28** (1982).
23. Gallo, R. C. *et al. Science* **220**, 865 (1983).
24. Gelmann, E. P. *et al. Science* **220**, 862 (1983).
25. Parks, W. *in Human T-cell Tumor Virus* (eds Gallo, R. C., Essex, M. & Gross, L.) (in the press).
26. Tedder, R. *et al.* Unpublished observations.
27. Groopman, J. E. *Nature* **308**, 769 (1984).

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100 years ago

In a crowded house on Tuesday last the Convocation of the University of Oxford passed the much-debated statute allowing women to enter for "certain of the honour examinations of the University." The statute has been opposed on very different grounds. The old Conservative Oxford School (fast becoming extinct among the resident teachers) of course objected to any change in favour of the higher education of women; with them went a portion for the High Church party, who look with disfavour on any proposal tending to bring women into intellectual competition with men. Others, again, opposed the statute on the ground that it was unfair to men, who have to keep certain terms and pass certain examin-

ations within a specified time if they wish to enter for an honour school, whereas the statute allows women to enter for honours without the same preliminary examinations, and without restrictions as to time and residence. Others again feared an influx of young ladies into Oxford, as likely to destroy the manliness of the undergraduates and spoil the natural modesty of the lady students. To these arguments the success which the present halls for ladies in Oxford have met with is the best answer. Their presence has not revolutionised the University; they have not been a stumbling-block to discipline nor a rock of offence to the Church. The women's examinations were on the same subjects, and the papers were set by the same men, as in the men's honour examinations before this statute passed. Now the same papers will serve for both, trouble will be saved, and the women who obtain honours will win a certificate universally recognised throughout the country. From *Nature* **30**, 19, 1 May 1884.

Ocean ecology

Fixation of CO₂ in dark midwater zones of the ocean

from Paul Tett

If the ocean began as a rich organic soup, it has since become so poor that many organisms have difficulty in getting enough energy and organic matter for growth and reproduction. The evolution of photoautotrophy must have been a breakthrough, allowing the light-driven uptake of simple inorganic compounds of carbon, nitrogen and phosphorus to supply the entire food needs of many planktonic plants. The illuminated superficial waters of the sea (the 'euphotic zone') are thus commonly seen as the main region of primary production, particularly by phytoplankton, whereas the deeper waters are seen as a zone of consumption in which heterotrophic animals and bacteria meet their energy demands by the oxidation of organic carbon supplied from the euphotic zone. On page 54 of this issue of *Nature*, Karl and his colleagues present evidence to suggest that bacteria in the midwater between the two zones can intercept the organic particles falling from the euphotic zone and, despite the absence of light, use the energy trapped in them to convert additional carbon dioxide into organic carbon (chemolithotrophy)¹.

As the earliest living organisms, prokaryotes probably exploited both inorganic and organic sources of energy and carbon until the development of the eukaryotes with their greatly improved efficiency of photosynthesis at high oxygen concentrations. That probably caused many of the remaining bacterial prokaryotes to specialize in heterotrophy — hence the traditional view of modern oceanic prokaryotes as degraders, consuming organic material originally produced by eukaryotic phytoplankton. Recent investigations of the smallest phytoplankton have, however, shown the importance of the prokaryotic cyanobacteria (once called 'blue-green algae') in oceanic primary production^{2,3}. And incorporation of carbon dioxide by thermophilic bacteria, using energy obtained by oxidizing locally enriched sulphide, iron or manganese, has been suggested as a source of primary production in food chains found near volcanic vents in deep water^{4,5}. From careful analyses of sedimenting material collected by free-floating traps at depths between 50 and 2,000 m in the eastern tropical Pacific Ocean south-west of Mexico, Karl *et al.* now add the suggestion that energy initially harnessed photoautotrophically by euphotic-zone phytoplankton for the incorporation of inorganic nitrogen (as well as carbon) can subsequently be chemolithotrophically used by midwater micro-

organisms to fix additional carbon dioxide¹.

The chemolithotrophic microorganisms involved are probably denitrifying bacteria using the ammonium produced by the action of heterotrophs on the organic material sinking down from the euphotic zone. By oxidizing this NH₄⁺ to NO₂⁻ the bacteria can obtain sufficient energy to take up carbon and reduce it to organic compounds. This chemolithotrophic use of nitrification to provide energy for carbon incorporation is less efficient than photosynthesis which, under optimal conditions, yields 0.08 moles of organic carbon for each einstein of light. Karl *et al.* cite yields of 0.03–0.06 moles of carbon fixed for each mole of ammonium–nitrogen oxidized, and estimate that potential chemolithotrophic production by microbes in their study region requires a maximum ammonium flux of about 0.8 mmol per m² per day. This flux, if completely supplied from photoautotrophically produced material with a carbon to nitrogen ratio of 7:1, requires an annual photosynthetic production of about 30 g carbon per m², close to lower estimates of tropical oceanic primary production. The greatest estimated chemolithotrophic production, about 0.4 mg carbon per m² per day, is equivalent to an annual average of 146 mg carbon per m², clearly only a small proportion of

phytoplankton production. The suggestion by Karl *et al.* that "the flux of energy out of the euphotic zone may greatly exceed that nominally contained in particulate organic detritus" thus over-emphasizes the importance of midwater chemolithotrophy, but these microbial processes may well influence the organization of midwater food webs.

Chemosynthesis has, of course, been known for many years and may, in unusual regions, such as the Black Sea, rival photosynthetic production⁶. Oceanic chemolithotrophs have ample supplies of their carbon source; their problem is to find at near-normal oxygen concentrations a sufficient concentration of energy source. In the Black Sea, chemosynthesis makes use of the high concentration of reduced material accumulated in the deoxygenated waters. The thermophilic bacteria of the deep-sea volcanic vents probably exploit local enrichments of reduced compounds^{4,5}. Perhaps the major novel contribution of Karl and co-workers is to document precisely a mechanism whereby chemolithotrophs can, through oxidizing ammonium associated with sinking particles, obtain sufficient energy for growth in the oligotrophic ocean. □

1. Karl, D.M., Knauer, G.A., Martin, J.H. & Ward, B.B. *Nature* **308**, 54 (1984).
2. Waterbury, J.B., S.W. Guillard, R.R.L. & Brand, L.E. *Nature* **277**, 293 (1979).
3. Platt, T., Subba Rao, D.V. & Irwin, B. *Nature* **301**, 702 (1983).
4. Jannasch, H.W. & Wirsen, C.O. *Bioscience* **29**, 592 (1979).
5. Karl, D.M., Wirsen, C.O. & Jannasch, H.W. *Science* **207**, 1345 (1980).
6. Sorokin, J.I. *J. Conseil* **29**, 41 (1964).

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Igneous petrology

Turbulence in petrology — the behaviour of komatiites

from E.G. Nisbet

CONSIDER the business of stamp-collecting: an elegant and harmless occupation of considerable economic benefit to countless post-offices, a source of endless diversion and speculation to the philatelist. Consider now the effect on that peaceful hobby of the discovery, somewhere to the east of Laputa, of a whole new continent filled with hitherto unknown national post-offices. Imagine the excitement and confusion of innumerable collectors scurrying in for examples. Eventually order is restored, what purports to be a comprehensive philatelic history is written and the hobby settles down to its normal delightful ways — until someone points out that some of the new stamps, though not exactly forgeries, are subtle but highly misleading overprints. But it is not clear how to identify them. Is history

to be rewritten? Confusion returns.

Igneous petrology may not quite be stamp-collecting but it, too, has its excitements, as when komatiites were discovered fifteen years ago. This sent many field workers scurrying to collect samples from which much has been learnt about the chemical properties of komatiites. Now Huppert *et al.*, on page 19 of this issue of *Nature*, cast doubt on some (but not all) of the current notions about the chemistry of komatiites from an examination of the physical properties of komatiite liquids¹. More reassuringly, they also provide some explanation of how komatiite lava flows can produce extraordinary large plate-like ('spinifex') crystals of olivine, thereby resolving a conflict between experimental results and cooling models.

When discovered, komatiites were a

wholly new class of extrusive igneous rock, rich in MgO and generally of Archaean age (older than 2.5×10^9 yr). Apart from providing some of the best clues to the composition, temperature and workings of the mantle of the early Earth, they are also of considerable economic value for their large nickel sulphide deposits. Komatiite lavas must have been very hot ($1,350$ – $1,650^\circ\text{C}$) and have had very low viscosity (0.05 – 1 Pas). Moreover, moving bodies of komatiitic lava (such as magmas in conduits or flows) must have had high Reynolds numbers and thus generally have been turbulent and capable of eroding walls or floors to conduits². From their detailed study, Huppert *et al.* show that komatiite lavas were typically turbulent, were accompanied by vigorous forced convection and that even for the narrowest fissures (0.3 m) their Reynolds number was probably far above the critical value for the onset of turbulence. Flow rates must have been very high: a 3-m-wide fissure would have had rates considerably greater than the largest eruptions in historic time.

In turbulent flow, mixing maintains a uniform temperature, heat transfer is much greater than in a laminar flow and, thus, the ground below the flow will heat up rapidly. Huppert *et al.* show that erosion by melting of this underlying ground would be rapid and significant. Assimilation of the melted ground would introduce up to 10 per cent contamination into the lava. Contamination would be of minor geochemical importance if the lava flowed over a ground made up of fresh sub-aerial komatiite flows; but if it flowed over hydrothermally altered flows, or over other rock types, or if it had arrived through a conduit lined with older rocks, the contamination could be of major significance. Fortunately for those making thermal models of the Archaean Earth, the MgO content of the lava (from which temperature estimates are principally derived) is not likely to have been changed much by contamination but the trace-element content could have been very substantially altered. Thus, radiogenic isotopes could be assimilated from an older country rock, leading to artefacts in dating. That may be the explanation for some anomalously old Sm–Nd dating of komatiites³. How reliable, then, is information from the incompatible trace elements, such as Ti, which has been used to construct geochemical models⁴ of the source mantle? Huppert *et al.* point out that contamination is likely to vary greatly from place to place within a flow so that any set of flows which displays non-systematic variation of trace elements with indices of olivine fractionation is likely to be contaminated. Contrarily, the very systematic variations from Belingwe samples⁵ may represent a pristine record (at least for some elements).

Huppert *et al.* also speculate about nickel sulphide deposits. Green and Naldrett suggested that some Canadian

and Western Australian deposits might have assimilated sulphur from nearby sulphide-rich banded ironstones⁶. Huppert *et al.* consider that lava may have digested such sediments by thermal erosion. Sulphur isotope studies may help determine the value of their hypothesis — which could be useful in selecting exploration targets. Finally, Huppert *et al.* consider spinifex textures — the long plates of olivine that hang down in sheaves from the top surfaces of some komatiite flows. Experimental cooling rates necessary to reproduce these textures are much higher than rates allowed by conductive cooling models but convective cooling in turbulent flows would explain the textures nicely and Huppert *et al.* have produced good analogues of spinifex textures in Na_2CO_3 solutions.

What then for us stamp collectors?

Clearly we must re-examine our data on the age and chemistry of komatiites and think again about the minor-element make-up of the mantle. Perhaps the recognition that contamination took place will help in resolving controversies about the extent of variation in chemistry of mantle source regions. More likely it will add to our uncertainty and send komatiitophiles scurrying back cheerfully to their field areas and to isotope laboratories. □

1. Huppert, H.E., Sparks, R.S.J., Turner, J.S. & Arndt, N.T. *Nature* **309**, 19 (1984).
2. Nisbet, E.G. in *Komatiites* (eds Arndt, N.T. & Nisbet, E.G.) Ch. 29, 513 (1982).
3. Chauvel, C. *et al.* *EOS* **64**, 330 (1983).
4. Smith, H.S. & Erlank, A.J. in *Komatiites* (eds Arndt, N.T. & Nisbet, E.G.) Ch. 22, 347 (1982).
5. Nisbet, E.G. *et al.* *J. Petrol.* **18**, 521 (1977).
6. Green, A.H. & Naldrett, A.J. *Econ. Geol.* **76**, 1503 (1981).

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Cell biology

A budding interest in the yeast cytoskeleton

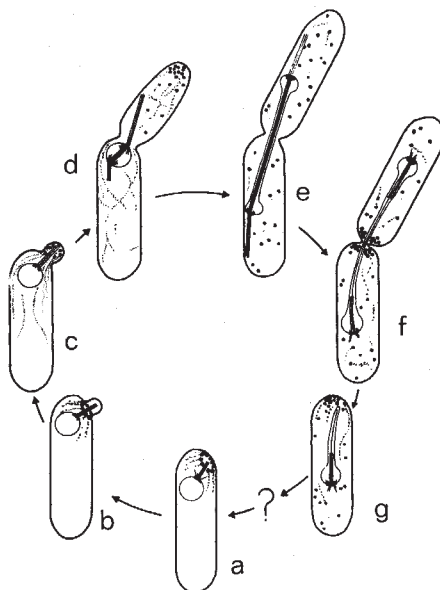
from Jeremy Hyams

COMPARED with the avalanche of papers devoted to the genetics and molecular biology of the budding yeast, *Saccharomyces cerevisiae*, which has sometimes threatened to bury the pages of this and some other journals over the past few years, yeast cell biology has had a fairly thin time of it. The trouble is that yeast cells are just not designed for the job — they are small, their cytoplasm is dense and, to make matters worse, they are surrounded

by a tough cell wall. Hence the curious situation that, whilst yeast actin has been purified and its gene cloned and sequenced¹, absolutely nothing has been known about its distribution or function during the budding cycle. Tubulin is somewhat better characterized, particularly in terms of the number and arrangement of spindle microtubules during cell division^{2–4}, but the role, and even the existence, of cytoplasmic microtubules has been far from certain. A recent pair of papers in *Journal of Cell Biology*^{5,6} therefore marks a major step forwards in yeast cytology.

What made this work possible was the isolation of monoclonal antibodies to yeast tubulin⁷. Anti-tubulin antibodies have been valuable tools for cell biologists for several years but conventional anti-tubulin antibodies raised against mammalian proteins do not cross-react with the microtubules of more primitive eukaryotes. On the other hand, the monoclonal antibodies to yeast tubulin appear to be universally cross-reactive. Using them and immunofluorescence microscopy, Kilmartin, Adams and Pringle have not only demonstrated the distribution of actin and tubulin during the yeast cell cycle in stunning detail but have also gone a long way to solving some of the old puzzles about the role of these proteins during budding^{5,6}.

Applied to the parent organism, the yeast anti-tubulin monoclonals generate intense staining both of the mitotic spindle and of extensive bundles of cytoplasmic microtubules (see the figure). In cells which have not yet formed a bud, cytoplasmic microtubules extend from the nucleus to the site from which the bud will emerge. Double staining with anti-actin and



Schematic representation of the changes in actin and tubulin distribution in budding yeast during the cell cycle (a–g). Microtubules appear as straight lines growing from the spindle pole body which is embedded in the persistent nuclear envelope. Actin is shown either as black dots or as fine fibres. From ref. 5.

fluorochrome-labelled phallotoxins, which bind specifically to F-actin, shows this region of the cytoplasm to be occupied by a ring of brightly staining actin dots. Simultaneous staining with Calcofluor reveals that the actin dots directly underly the ring of chitin which is laid down in the otherwise chitin-free cell wall and which defines the budding site.

As the bud emerges and enlarges the actin dots are displaced towards the bud tip at the same time as fine fibres of actin extend through both the bud and the mother cell. During this reorganization the spindle forms and elongates. Significantly, in view of the current speculation regarding the role of actin in chromosome movement, no actin is detected within the nucleus at any stage of the budding cycle of yeast. When the bud has reached its maximum dimensions the actin dots disperse through the cytoplasm but then concentrate in the neck region at the time of cytokinesis.

The changes in the distribution of actin are consistent with the suggestion that actin has a role in the localized desposition of cell-wall materials, first in the chitin ring and later at the tip of the bud. A similar pattern of staining in another fungus,

Uromyces phaseoli, led to the suggestion that the dots of actin were microvesicles coated with filaments having the same dimensions as F-actin ('filasomes')⁸. In *S. cerevisiae*, cytoplasmic microtubules may direct similar vesicles, containing cell-wall precursors, to their sites of deposition² or may simply serve to orient correctly the spindle for its later extension into the bud.

The introduction of effective immunofluorescence procedures to *S. cerevisiae* adds the yeasts to the list of organisms in which fundamental cell-biological problems can now be studied. Remembering the sophisticated genetics that has already been developed for yeasts, the potential of these simple organisms seems to be limitless. □

1. Ng, R. & Abelson, J. *Proc. natn. Acad. Sci. U.S.A.* **77**, 391 (1980).
2. Byers, B. & Geotsch, L. *J. Bact.* **124**, 511 (1975).
3. Peterson, J.B. & Ris, H. *J. Cell Sci.* **22**, 219 (1976).
4. King, S.M., Hyams, J.S. & Luba, A. *J. Cell Biol.* **94**, 341 (1982).
5. Kilmartin, J.V. & Adams, A.E.M. *J. Cell Biol.* **98**, 922 (1984).
6. Adams, A.E.M. & Pringle, J.R. *J. Cell Biol.* **98**, 934 (1984).
7. Kilmartin, J.V., Wright, B. & Milstein, C. *J. Cell Biol.* **93**, 576 (1982).
8. Hoch, H.C. & Staples, R.C. *Eur. J. Cell Biol.* **32**, 52 (1983).

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Cosmology

Fluctuations in microwave background radiation

from M.S. Longair

NEARLY twenty years after the discovery of the microwave background radiation by Penzias and Wilson, all but the most sceptical cosmologists are convinced that the hot big-bang model provides the most promising framework within which to study the more profound problems of cosmology. It has long been recognized that directional fluctuations (ΔI) in the intensity (I) of the microwave background radiation should be a powerful way of investigating a wide range of properties of the hot big-bang model. The detection of such fluctuations has, however, proved to be extremely difficult and it is only with the paper of Birkinshaw, Gull and Hardeback on page 34 of this issue that it is possible to say with confidence that at least one type of small-scale fluctuation has been observed.

The only anisotropic signal unambiguously detected so far at a level of about $\Delta I/I \sim 10^{-3}$ is associated with the motion of the Earth relative to the frame of reference in which the background is isotropic. Otherwise there are only upper limits to the intensity fluctuations, the most sensitive at a level of about $\Delta I/I \sim 10^{-4}$. The problem is that, at this level of sensitivity, very small instrumental effects can introduce spurious fluctuations while, in addition, the atmosphere introduces fluctuations due to astronomical 'seeing'

in the radio waveband.

Birkinshaw *et al.* have searched for the small dips or decrements in the microwave background radiation first predicted by Sunyaev and Zel'dovich in 1968 on the grounds that the spectrum of the microwave background could be distorted by quantities of hot gas along the line of sight. If the gas were very much hotter than the black-body radiation, the hot electrons would heat the radiation by Compton scattering and, in a simple approximation, the whole microwave background spectrum would be shifted to a slightly higher frequency while the total number of photons will be unchanged. Thus, if the microwave background radiation traverses a region of hot gas on its way to the observer, there will be a decrease in the intensity in the long-wavelength (Rayleigh-Jeans) region of the Planck spectrum and an increase in the high-frequency (Wien) region.

Since X-ray observations with the Uhuru, Ariel V and Einstein satellite observatories have shown that there are vast quantities of hot gas at temperatures of 10^7 – 10^8 K in the direction of rich clusters of galaxies, Birkinshaw *et al.* turned the 40-m telescope of the Owens Valley Radio Observatory, California, in those directions in their search for intensity

fluctuations. As a result, after years of marginal detections and several false starts, fluctuations at a high level of significance have been observed in the direction of three rich clusters of galaxies. The relative intensities of these fluctuations range from about 5×10^{-4} in the case of cluster 0016 + 16 to about 2×10^{-4} in the case of the Abell clusters 665 and 2218. It is particularly impressive that these values are all claimed at or above the 7 σ confidence level — a striking measure of the sensitivity of the observations.

Astrophysically, this result is important because the fluctuations provide information about the hot gas which has modulated the microwave background in some particular direction in the sky. Indeed, the magnitude of the decrement is determined solely by the optical depth for Compton scattering by the hot gas, which is proportional to the electron density N_e , the electron temperature T_e and the dimension of the cloud in the line-of-sight. Complementary information about the hot gas can be found from X-ray observations of the clusters. The temperature can be obtained from the shape of the spectrum, which for energetic X rays is roughly proportional to $\exp(-h\nu/kT_e)$ while the total intensity of X-ray emission is proportional to N_e^2 , to $T_e^{-1/2}$ and to the volume of the cloud.

Thus, if adequate spatial resolution is available at radio and X-ray wavelengths and the distribution of gas in the cluster is reasonably well behaved, the physical conditions are overdetermined so that the physical size of the gas cloud in a cluster can also be found. That, in turn, opens up the exciting prospect of a new route to the determination of Hubble's constant since we now know the physical size of a 'measuring rod' at a known redshift.

But this achievement is only the beginning of what Birkinshaw and his colleagues foresee as a new epoch in observational cosmology. The Sunyaev-Zel'dovich effect was probably the easiest of challenges; if sensitivity can be improved by an order of magnitude, a whole new range of phenomena will come within our observational grasp.

Among the most important goals is the discovery of fluctuations predicted to be associated with the initial collapse of proto-galaxies and protoclusters. According to all acceptable theories of the origin of structure in the Universe, the growth of the fluctuations of ordinary matter from which galaxies and clusters have formed became possible only after matter and radiation were decoupled at a redshift of about 1,500, corresponding to an epoch of 10^6 – 10^7 years after the big bang. The requirement that galaxies and clusters should now exist results in lower limits to the material density fluctuations at that early epoch and thus, because of Compton scattering, to radiation anisotropies that should now be apparent as fluctuations in the microwave background in the range

$\Delta I/I \sim 10^{-4} - 10^{-5}$. Observations of such fluctuations would provide strong support for the hypothesis that structures formed by collapse which began when matter and radiation decoupled. This is a rather general argument which remains valid even in recent scenarios of galaxy formation involving massive neutrinos and other weakly interacting particles that may contain most of the mass of the Universe.

Other sources are expected to produce fluctuations at about the same level. Thus the reheating of gas at a late epoch should induce fluctuations, while the passage of radiation through large collapsing structures can cause fluctuations by the gravitational redshifting produced in the process. Even the hot gas in rich clusters, now detected by Birkinshaw *et al.*, must

yield fluctuations on a fine scale, and these may well limit the detectability of genuine primordial fluctuations.

Such an approach is, nevertheless, one of the few that can provide observational information about the epochs between about 10^7 and 2×10^9 years. Already, the Cambridge radioastronomers have developed proposals for achieving the extra factor of 10 in sensitivity needed to tackle these more ambitious programmes. It is hard to disagree with their view that the next decade will be a period when these exciting and fundamental observations become an integral part of astrophysical cosmology. □

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Astronomy

Black holes, accretion discs and Seyfert galaxies

from C. Martin Gaskell

A LARGE consortium of European astronomers has recently published the third instalment of results of their ongoing programme monitoring the nearby Seyfert 1 galaxy NGC4151 with the International Ultraviolet Explorer satellite (IUE)¹. Results have already been published from similar programmes monitoring other Seyferts (the lowest-luminosity quasars) but NGC4151, the first Seyfert to be discovered², has always received more ground- and space-based attention than any other. Considerable publicity before publication by the Royal Greenwich Observatory alleged that the 'mass of the black hole' in NGC4151 had been measured. Although this claim captured the imagination of the British press, the truth is that neither the IUE consortium nor anybody else has proved that there is a black hole in NGC4151 or any other quasar-like object. The mass estimated for the central object is in fact not new. An almost identical value was derived 25 years ago³ and the basic method employed has already been used innumerable times over the last couple of decades. It is widely recognized that the method could be giving a wrong answer however. The IUE consortium claims to have made 'remarkable progress' in understanding the nuclear energy source of NGC4151, but, interesting as their observations are, unfortunately they neither prove nor disprove the current standard theory that quasars and similar objects are empowered by accretion of matter in a disc onto a massive black hole. Much of the interpretation of changes in the ultraviolet spectrum is probably wrong.

How do you estimate the mass of a quasar nucleus? The most direct way is to use Newton's law of gravity. Since one obviously cannot follow a single test particle

around an orbit one has to use the integrated properties of the ensemble of cloudlets in the broad-line region and make certain assumptions. The most usual assumption is that the cloudlets are in bound orbits with an isotropic velocity distribution. To deduce the central mass one needs to know the average velocity and the average distance. The velocities of broad-line clouds have been well known since the pioneering work of Seyfert⁴ in 1943. The Doppler widths of the broad lines imply velocities of several per cent of the speed of light. The sizes of broad-line regions were first calculated from photoionization considerations^{3,5} but the subsequent discovery, in 1967, of variability of emission lines in Seyferts^{6,7} permitted an independent estimate of the size of the broad-line regions from light-travel-time considerations⁸. The two methods are in satisfactory agreement. It is the latter method that Ulrich *et al.*¹ have used. Woltjer³ estimated the central mass of NGC4151 to be 3×10^7 solar masses as long ago as 1959 and this sort of mass has been almost universally accepted by workers in the field ever since. Clearly the IUE observers have not made a new contribution here.

Are we confident that we know the mass of the central object fairly well? The answer is no. The problem with the standard newtonian calculation described above is that the assumption of bound orbits (or at least of cloud motions dominated by gravity) is not valid. There is abundant evidence for the outflow of gas from quasars and Seyferts in general (this has already been reviewed in a recent *News and Views*⁹) and in NGC4151 in particular¹⁰. If the cloud motions are dominated by radiation pressure (causing the outflow), rather

than by gravity, the assumption of orbital motion is clearly invalid and the mass can easily have been overestimated or underestimated by an order of magnitude.

Why do we think that quasars contain massive black holes? All the arguments for the existence of massive black holes in active galactic nuclei are theoretical. If the mass of the central object is indeed 3×10^7 solar masses this does not automatically prove that it is a black hole rather than, say, a supermassive star or a spinar. Nonetheless, accretion of matter onto a massive black hole has long been considered the most likely source of energy in quasars¹¹. If the accreting matter has any angular momentum at all it will collapse into a flattened configuration (an 'accretion disc'). Such discs are known to exist around many collapsed stellar objects (the dwarf novae, cataclysmic variables and so on) in our own Galaxy. The standard model of quasars is currently an accretion disc surrounding a massive black hole.

Although such discs are receiving considerable theoretical attention, the only observational hint that they exist around quasars is a slight curvature in the spectral energy distribution in the optical-ultraviolet region. Shields¹² has proposed that this could be due to thermal emission from an accretion disc superimposed on an underlying power-law non-thermal continuum. But this is by no means the only interpretation of the continuum energy distribution in quasar spectra. The interpretation is complicated by the presence of many weak blended Fe II lines¹³ and uncertainties in the amount of reddening by dust in the quasars. The excess emission around 3,000 Å (the so-called '3,000 Å bump') is almost certainly Balmer continuum and blended Fe II line emission^{14,15}. The remaining curvature in the ultraviolet-optical spectra of quasars can be understood in terms of standard synchrotron-self-Compton theory without invoking any additional thermal component at all¹⁶. If there is a thermal excess it does not have to be from an accretion disc — it could be from the

1. Ulrich, M.-H. *et al. Mon. Not. R. astr. Soc.* **206**, 221 (1984).
2. Campbell, W.W. & Moore, J.H. *Lick Ob. Pub.* **13**, 122 (1918).
3. Woltjer, L. *Astrophys. J.* **130**, 38 (1959).
4. Seyfert, C.K. *Astrophys. J.* **97**, 28 (1943).
5. Setti, F. & Woltjer, L. *Astrophys. J.* **144**, 838 (1966).
6. Dibal, E.A. & Pronik, V.B. *Astron. Zh.* **44**, 838 (1966).
7. Andriulat, Y. & Souffrin, S. *Astrophys. Lett.* **1**, 111 (1968).
8. Cherepashchuk, A.M. & Lyuti, V.M. *Astrophys. Lett.* **13**, 165 (1973).
9. Gaskell, C.M. *Nature* **307**, 210 (1983).
10. Anderson, K.S. & Kraft, R.P. *Astrophys. J.* **158**, 859 (1969).
11. Salpeter, E.E. *Astrophys. J.* **140**, 769 (1964).
12. Shields, G.A. *Nature* **272**, 706 (1978).
13. Wills, B.J. *et al. Astrophys. J.* **237**, 319 (1980).
14. Gaskell, C.M. thesis, Univ. California, Santa Cruz (1981).
15. Wills, B.J. *Liège astrophys. Colloq.* **24**, 458 (1983).
16. Puettner, R.C. *et al. Astrophys. J.* **257**, 487 (1982).
17. Sorrell, W.H. *Nature* **291**, 394 (1981).
18. Ulrich, M.-H. *et al. Mon. Not. R. astr. Soc.* **192**, 561 (1980).
19. Clavel, J., Joly, M., Collin-Souffrin, S., Bergeron, J. & Penston, M.V. *Mon. Not. R. astr. Soc.* **202**, 85 (1983).
20. Barr, P., Willis, A.J. & Wilson, R. *Mon. Not. R. astr. Soc.* **202**, 453 (1983).
21. Gaskell, C.M. *Liège astrophys. Colloq.* **24**, 473 (1983).

photosphere of a massive superstar¹⁷ or from very-high-density broad-line-region gas close to the central object¹⁸.

Observations of time variability of the continuum spectral shape of Seyferts and quasars are going to be important in understanding the origin of the continuum and the observations by Ulrich *et al.*¹ and others^{19,20} have shown interesting changes in the ultraviolet continuum of NGC4151 and other Seyferts. IUE observations alone, however, just do not provide enough wavelength coverage and the changes reported are not, I believe, true changes in the continuum shape but rather changes in the relative intensity of the Fe II emission at 3,000 Å (that is, the 3,000 Å bump). This is probably the cause of the otherwise puzzling result of Ulrich *et al.*¹ that the C IV 1,549 Å emission-line variability is better correlated with the continuum emission at 2,500 Å than with the continuum further into the ultraviolet

(that is, nearer to the relevant ionization edges), and other inconsistencies which they note. To measure the true continuum one needs to cover many octaves in frequency and it is to be strongly hoped that future IUE observations of NGC4151 (and other Seyferts) will be scheduled with simultaneous longer-wavelength optical and infrared observations (and for that matter X-ray observations as well).

Although our picture of NGC4151 and other active galactic nuclei is still very fuzzy and little has actually been proved, it must be said that everything is still consistent with such objects having at least one massive black hole in their centres (and some may have two²¹). Similarly there is no evidence so far that quasars do not have accretion discs powering them, but it would be nice to have proof that they do. □

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Protein structure

Oxygen carriers of a third kind

from K.E. van Holde

HAEMOGLOBIN is not the only way to transport oxygen in a circulatory system. Some invertebrate phyla use altogether different proteins to serve this function. These include the haemerythrins, utilized by sipunculids, brachiopods and a few other invertebrates, and the haemocyanins, found in a wide variety of molluscs and arthropods. The structures of many haemoglobins, and even one haemerythrin¹, have been determined to high resolution by diffraction techniques, but until the paper of Gaykema *et al.*² on page 23, such information has been wholly lacking for the haemocyanins. This hiatus has seemed almost paradoxical, for the haemocyanins were the first proteins to be studied in detail by biophysical techniques, notably in some of the earliest ultracentrifuge studies of Svedberg and his collaborators³.

The problem of how to bind oxygen in a way that is reversible receives a radically different solution in haemocyanins than it does in haemoglobins and haemerythrins. Rather than using iron as an oxygen ligand, haemocyanins employ pairs of antiferromagnetically coupled copper ions^{4,5}. Such copper pairs are found in a number of other proteins, but for none of these is the detailed structure known. Thus the determination, to a resolution of 3.2 Å, of the structure of the haemocyanin of the spiny lobster, *Panulirus interruptus*, is of major importance.

The native molecule, which is one of the simpler haemocyanins, exists as a hexamer of two kinds of very similar subunits, each of molecular weight (MW) about 75,000. The entire hexameric structure has now been determined by Gaykema *et al.*²,

making it one of the largest proteins to be analysed by X-ray diffraction. The subunits are found to be arrayed with 32 symmetry; two trimers, related by 2-fold axes, are stacked face to face. Each subunit consists of three domains of approximately equal size. The second of these domains carries the copper pair. Each copper ion is bound by three residues, two of which are definitely histidines. The third ligand is probably also a histidine, but confirmation will require further sequencing.

The structure determined by Gaykema *et al.* seems to be that of deoxyhaemocyanin but studies of a subunit of *Limulus* oxyhaemocyanin are under way in the laboratory of W. Love, at Johns Hopkins University⁶. It will be of considerable interest to see how the oxy- and deoxy-structures compare, for haemocyanins are allosteric proteins, exhibiting both cooperativity in oxygen binding and heterotropic allostery, including a pronounced Bohr effect. So far, our only model for these functions has been haemoglobin (haemerythrin appears to be non-cooperative⁷); it is conceivable that similar effects will be found to be produced by quite dissimilar mechanisms in a protein as different as haemocyanin. This would represent an interesting example of convergent evolution of protein function.

The structure found for the haemocyanin seems wholly unrelated to that of haemerythrin or any haemoglobin, so haemocyanin must have a different ancestry. Is it shared with tyrosinases, ceruloplasmin and laccase, all proteins with a similar binuclear copper centre? Although the three-dimensional structures of these proteins are unknown, their amino

acid sequences have certain motifs in common, suggesting similar modes of copper binding. In all three proteins there exist pairs of histidines separated by three amino acids, resembling those now identified among the copper ligands of haemocyanin. Confirmation of the hypothetical ancestry may be difficult, however, for the haemocyanins have presumably been evolving independently for something on the order of 500 million years. Indeed, a comparison of the complete sequence of a spider haemocyanin with *Neurospora* tyrosinase gave no evidence of homology⁸.

Gaykema *et al.* point out a peculiar aspect of the *Panulirus* haemocyanin structure. Its carboxy-terminal domain contains a seven-stranded antiparallel β -barrel, remarkably similar in topology to the β -barrels in (Cu-Zn) superoxide dismutase and immunoglobulins⁹. Since the amino acid sequences are entirely different, they argue that this may simply be a very stable structure which has evolved independently in different proteins.

A final puzzle, which may be clarified by further structural studies, is the relationship between arthropod and molluscan haemocyanins. The oxygen-binding site of both types seems quite similar but their gross molecular architecture is entirely different. All known arthropod haemocyanins are built of 75,000-MW chains, arranged in hexamers, as in *Panulirus*. These hexamers are often combined to form even larger structures. On the other hand, the polypeptide chains of molluscan haemocyanins are much larger; units of MW about 400,000, containing eight globular domains and eight oxygen-binding sites, are commonly observed^{4,5}. Gaykema *et al.* suggest that a molluscan domain of ~50,000 MW may correspond to two of the three 25,000-MW domains observed in the *Panulirus* structure, one carrying a binuclear copper site. Thus, it is conceivable that molluscan and arthropod haemocyanins arose via separate evolutionary events from some common copper-protein ancestor.

The new structural studies, combined with a number of sequence determinations now underway, promise to broaden our perspectives concerning the mechanisms that organisms have evolved to transport oxygen. □

1. Ward, K. B. *et al.* *Nature* **257**, 818 (1975).
2. Gaykema, W. P. J. *et al.* *Nature* **308**, 23 (1984).
3. Svedberg, T. & Pedersen, K. O. *The Ultracentrifuge* (Oxford University Press, 1940).
4. van Holde, K. E. & Miller, K. I. *Rev. Biophys.* **15**, 1 (1982).
5. Ellerton, H. D. *et al.* *Prog. Biophys. molec. Biol.* **41**, 143 (1983).
6. Love, W., Padlan, E. & Magnus, K. Personal communication.
7. Magnus, C. P. & Kondon, M. *Comp. Biochem. Physiol.* **50A**, 777 (1975).
8. Schneider *et al.* *Hoppe-Seyler's Z. physiol. Chem.* **364**, 1357 (1983).
9. Richardson *et al.* *J. molec. Biol.* **102**, 221 (1976).

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Emplacement and cooling of komatiite lavas

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We propose that komatiite lavas were emplaced as turbulent flows, accompanied by vigorous forced convection with cooling rates often in excess of hundreds of °C h⁻¹. They melted and assimilated up to 10% of the ground over which they flowed, forming deep channels. Nickel sulphide mineralization may have resulted from incorporation of sulphur-rich sediment. After emplacement, high cooling rates persisted, resulting in spinifex textures due to compositional convection.

KOMATIITES are believed to represent ultramafic liquids which erupted at temperatures of 1,400–1,700 °C with estimated viscosities in the range 0.1–1 Pa s, and with MgO contents of 18–35%^{1–3}. Such lavas are mostly of Archaean age. They indicate that thermal conditions of the early Earth were significantly different from those of today and provide insights into the composition of the Archaean mantle. They commonly exhibit spectacular spinifex textures and internal layering⁴, and some are also the host to important nickel sulphide mineralization⁵. Interpretation of their chemical compositions, petrological and geological features and mineralization requires an understanding of their eruption, emplacement and cooling. As a contribution to this understanding, we present both a fluid dynamical analysis of the flow and cooling of komatiites and experimental studies which simulate the formation of spinifex texture in ponded komatiite lavas.

Recent basalts have eruption temperatures usually below 1,250 °C and viscosities >50 Pa s; almost all of them flow by laminar shear at low Reynolds number and cool by conduction. We propose that, because of their much lower viscosities, komatiites would have flowed turbulently at high Reynolds number, and would have cooled initially by forced convection. We suggest that, after emplacement, komatiite lavas still cooled rapidly by thermal convection and demonstrate, by experimental studies on cooling of saturated aqueous solutions, that convective effects can strongly influence the development of the characteristic spinifex textures.

Magma ascent

Magma ascends to the surface because of its buoyancy^{6,7}. At high Reynolds number, Re , the two-dimensional discharge rate, Q , from a long fissure of width d can be estimated as

$$Q = \left(\frac{g\Delta\rho}{k\rho_m} \right)^{1/2} d^{3/2} \quad (1)$$

where g is gravity, $\Delta\rho$ is the assumed constant density difference

between the magma and the lithosphere overlying its source, ρ_m is the magma density, k is a friction coefficient which can assume values between ~0.01 and 0.06 depending on the surface roughness of the fissure⁷, and the Reynolds number is

$$Re = Q/\nu \quad (2)$$

where ν is the kinematic viscosity. In principle, in order to account for the density variation in the lithosphere, the compressibility of the melt and the tortuous nature of the fissure, a more elaborate treatment than that leading to equation (1) is required. However, the accuracy of equation (1) is sufficient for our calculations. At shallow depths in the lithosphere, the value of $\Delta\rho$ is ~300 kg m⁻³, whereas at depths of 100 km the value of $\Delta\rho$ has been estimated as 100 kg m⁻³ (ref. 8).

Table 1 gives the values of Q , flow velocity $v = Q/d$, and Re for a plausible range of natural fissure widths. The komatiite is assumed to have a viscosity of 0.3 Pa s, a density of 2,800 kg m⁻³ and we have set $\Delta\rho$ to a conservative value of 100 kg m⁻³. The value of 0.03 has been used for the friction coefficient. Table 1 shows that even for the narrowest fissure (0.3 m), Re greatly exceeds the critical value (equal to ~2,000) required for turbulent flow. Komatiites are thus likely to have flowed turbulently up the fissure before eruption. This should be compared with the largest historic eruptions of basalts, for which Q and Re are less than 10 m² s⁻¹ and 600 respectively⁷.

Lava emplacement

A fundamental feature of a turbulent flow is that the heat transfer is much greater than in a laminar flow, where heat is lost entirely by conduction. In turbulent flows, mixing maintains a uniform temperature throughout the span of the flow and greatly increases heat transfer to the surroundings. Thus, in turbulent komatiite flows the ground surface would be quickly heated up to temperatures of 1,600–1,700 °C. In greenstone belts, komatiites often flow over rocks, such as tholeiitic basalt and sediment^{9,10}, which have substantially lower melting temperatures. We suggest that komatiites could have melted and assimilated this underlying rock.

Our model, a geometric sketch of which is presented in Fig. 1, is related to that proposed by Hulme^{11,12}, who argued that the sinuous rilles on the Moon were caused by thermal erosion of lava channels by turbulent lunar flows. We consider flow on a shallow slope beneath a deep body of water. Both floor rocks and water have initial temperature T_0 , which is taken to be 0 °C. The heat transfer from the komatiite, due to forced, turbulent convection, melts some of the ground which is then

Table 1 The two-dimensional flow rate, Q , the vertical velocity, v , and the Reynolds number, Re in a fissure of width d

d (m)	Q (m ² s ⁻¹)	v (m s ⁻¹)	Re
0.3	0.56	1.9	5.2×10^3
1.0	3.4	3.4	3.2×10^4
3.0	17.8	5.9	1.6×10^5
10.0	108	10.8	1.0×10^6

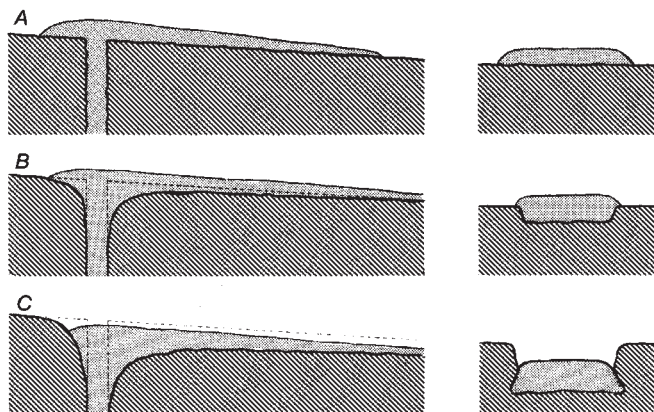


Fig. 1 Longitudinal and cross-sectional sketches of the fissure and lava flow showing the melting of the ground and the formation of an erosional channel with time.

assimilated. Efficient cooling by the water above the flow forms a thin crust on top of the lava and prevents any mixing between the two. As the lava cools, crystals form within it, which decreases the rate of cooling. The komatiite is assumed to flow in the form of a gravity current of high Reynolds number over a slope which is sufficiently small that its effects can be neglected. The thickness of the current, h , can then be determined¹³ by

$$h = (\frac{1}{2} Q^2 \rho_l / g \delta \rho)^{1/3} \quad (3)$$

where $\delta \rho$ represents the density difference ($1,800 \text{ kg m}^{-3}$) between the komatiite and seawater. Table 2 presents the flow thickness, velocity, u , and Reynolds number for different values of Q . A gravity current with a Reynolds number greater than ~ 500 is fully turbulent¹⁴. The values in Table 2 indicate that this is the case for all komatiite flows.

At steady state the melting rate is given by^{11,15}

$$\frac{dS}{dt} = \frac{H}{\rho_g [c_g (T_m - T_0) + L_g]} \quad (4)$$

where H is the heat transfer rate to the ground, ρ_g is the density of the ground, c_g is the specific heat of the ground, T_m is both the melting temperature of the ground and the solidification temperature of the komatiite and L_g is the heat of fusion of the ground. The heat transfer is related to T_1 , the lava temperature, and T_m through a heat transfer coefficient, h_T , by¹⁵

$$H = h_T (T_1 - T_m) \quad (5)$$

An approximation to the heat transfer coefficient can be obtained by modifying empirical relationships for turbulent pipe flows¹⁵. This leads to

$$h_T = 0.018 \frac{k_l}{h} Pr^{0.4} Re^{0.8} \quad (6)$$

where k_l is the lava conductivity and Pr its Prandtl number.

Table 2 shows that transfer coefficients of between 500 and $1,000 \text{ W m}^{-2} \text{ } ^\circ\text{C}^{-1}$ result for flow rates in the range 0.5–

Table 3 The cooling rate at the source, $\dot{T}(0)$, the erosion rate at the source, $\dot{S}(0)$, and the length scale over which the temperature of the lava decreases, D^{-1} , as a function of Q

$Q(\text{m}^2 \text{ s}^{-1})$	$\dot{T}(0) (^\circ\text{C h}^{-1})$	$\dot{S}(0) (\text{m per day})$	$D^{-1} (\text{km})$
1	1,300 (2,730)	3.33 (6.98)	2.9 (1.4)
10	280 (590)	3.33 (6.98)	28.5 (13.6)
100	61 (127)	3.33 (6.98)	285 (136)

The second entry, in parentheses, is the value if latent heat effects are set equal to zero.

$100 \text{ m}^2 \text{ s}^{-1}$. This variation in the heat transfer coefficient is fairly small and for simplicity we have taken $h_T = 750 \text{ W m}^{-2} \text{ } ^\circ\text{C}^{-1}$ in the subsequent calculations. With the values of physical parameters listed below inserted into equations (4) to (6), the melting rate of the ground near the source is calculated to be $8.1 \times 10^{-5} \text{ m s}^{-1}$ (7 m per day), confirming that thermal erosion was a major effect during the emplacement of komatiites. (The physical parameters used were: $k_l = k_c = 1 \text{ W m}^{-1} \text{ } ^\circ\text{C}^{-1}$; $c_l = c_g = 730 \text{ J kg}^{-1} \text{ } ^\circ\text{C}^{-1}$; $\rho_l = 2,800 \text{ kg m}^{-3}$; $\rho_g = 2,700 \text{ kg m}^{-3}$; $L_g = 5 \times 10^5 \text{ J kg}^{-1}$; $T_0 = 0^\circ\text{C}$; $T_m = 1,200^\circ\text{C}$; $T_1 (x=0) = 1,600^\circ\text{C}$; $\theta_0 = T_1 (x=0) - T_m = 400^\circ\text{C}$; and $Pr = 219$.)

A thin crust forms at the contact with seawater. At the top of the crust the temperature is T_0 , while at the bottom it is T_m . The crustal thickness, a , is determined from the fact that in a steady state the conductive heat transfer through the crust equals the heat transfer from the lava given by equation (5). Thus

$$a = k_c (T_m - T_0) / h_T (T_1 - T_m) \quad (7)$$

where k_c is the conductivity of the crust. Near the source $a \approx 4 \text{ mm}$ and increases away from the source as the lava temperature decreases.

Cooling of the lava

Heat loss from the lava causes crystallization, which releases latent heat, L_x . A quantitative heat budget calculation indicates that the lava temperature as a function of downstream distance x is given by

$$T_1 = T_m + \frac{D\theta_0}{(E\theta_0 + D) e^{Dx} - E\theta_0} \quad (8)$$

where θ_0 is the difference between the temperature of the lava at source and the melting temperature of the ground, $D = 2h_T^x / (\rho_l c_l Q)$,

$$E = \frac{h_T^x}{Q\rho_g [c_g (T_m - T_0) + L_g]} \quad (9)$$

and

$$h_T^x = \frac{h_T}{1 - L_x x'(T) / c_l} \quad (10)$$

with $x'(T) = -\frac{1}{625} \text{ } ^\circ\text{C}^{-1}$ representing the rate of change of crystal fraction with temperature¹⁶. For $Q = 10 \text{ m}^2 \text{ s}^{-1}$, the lava temperature as a function of distance is plotted in Fig. 2A. The temperature decreases with a length scale given by D^{-1} , which is evaluated for other flow rates in Table 3. If the lava increases the ground temperature to T_m but does not melt it, its temperature is given by $T_1 = T_m + \theta_0 e^{-Dx}$, also plotted in Fig. 2A.

The cooling rates of the lava flow for $Q = 10 \text{ m}^2 \text{ s}^{-1}$ are plotted in Fig. 2B; one curve incorporates latent heat release due to crystallization while the other does not. With latent heat effects the cooling rate is 280°C h^{-1} near the source and decreases steadily away from it. Initial cooling rates for other flow rates are given in Table 3.

These calculations assume that crystallization occurs in thermodynamic equilibrium, although experimental studies^{17,18} on magnesian-rich basic melts show that rapid cooling rates can

Table 2 Lava flow thickness, h , its velocity, u , the Reynolds number, Re , the heat transfer coefficient, h_T , and the coefficient modified by crystallization, h_T^x , as a function of Q

$Q(\text{m}^2 \text{ s}^{-1})$	$h(\text{m})$	$u(\text{m s}^{-1})$	Re	$h_T (\text{W m}^{-2} \text{ } ^\circ\text{C}^{-1})$	$h_T^x (\text{W m}^{-2} \text{ } ^\circ\text{C}^{-1})$
0.5	0.27	1.8	4.7×10^3	498	238
1	0.43	2.3	9.3×10^3	543	259
10	1.99	5.0	9.3×10^4	739	353
100	9.26	10.8	9.3×10^5	1,000	477

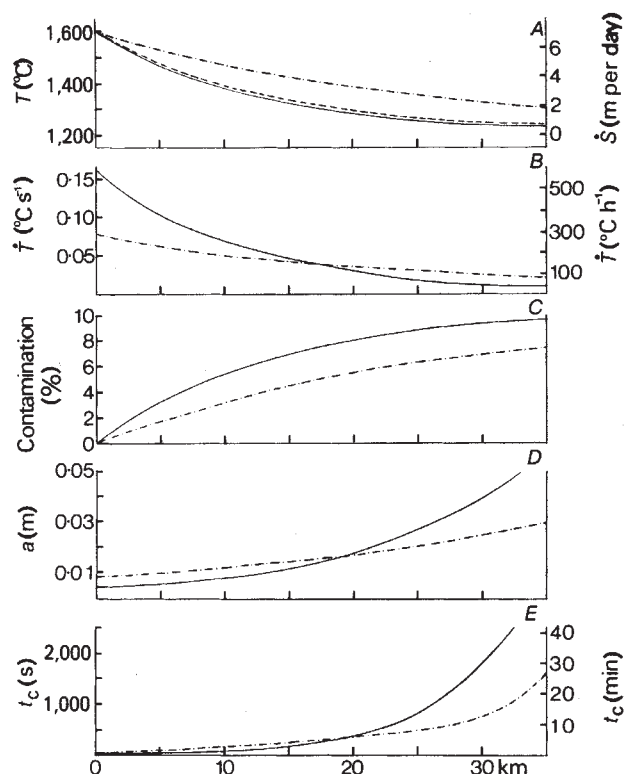


Fig. 2 A, The temperature of the lava, T , and the erosion rate of the ground, S . —, With melting; ---, without melting. B, The cooling rate; C, the percentage of contamination; D, the crustal thickness, a ; and E, the time scale for the crust to form; all as a function of distance from the source. In A–E the dot-dash (– · –) line incorporates the latent heat release due to crystal formation, while the solid line omits this effect ($L = 0$).

cause large undercoolings. Extrapolation of Donaldson's experimental data¹⁸ to cooling rates of several hundreds of °C per hour suggests undercoolings of 50–70 °C. The cooling rates in excess of 10^3 °C h⁻¹, for small Q , might result in considerable delays in olivine nucleation, with the extreme case of zero crystallization. Table 3 shows the results of calculations neglecting crystallization completely; cooling rates and melting rates increase as the undercooling increases.

As the melting rate of the ground is proportional to $T_1 - T_m$, it has the same functional form as T_1 (Fig. 2A). Our calculations indicate that one hour after eruption, with the flow rate $Q = 10 \text{ m}^2 \text{ s}^{-1}$, the channel depth at source would be 14 cm, while 15 km from the source it would be 8 cm. After a week the depth at source would be 23 m and 15 km from source it would be 13 m. If no crystallization occurred, the depths would be just over twice these values. If the lava flow were 250 m wide, the volume erupted after one hour and one week would be 9×10^6 and $1.5 \times 10^9 \text{ m}^3$, respectively. These volumes are comparable to small- and medium-sized basalt eruptions.

Assimilation of the melted ground contaminates the lava, as indicated in Fig. 2C. The contamination rises steadily to an asymptotic value of 10.4%, independent of the flow rate, and could significantly change the trace element and isotopic composition of the lava, although it would have only minor effects on the major element composition.

The crustal thickness, given by equation (7) and plotted in Fig. 2D, is of the order of a few centimetres, consistent with the geological evidence that komatiites often show an upper quench glassy zone of this magnitude⁴. The time scale for this crust to grow (Fig. 2E) is quite short and indicates that if at any time

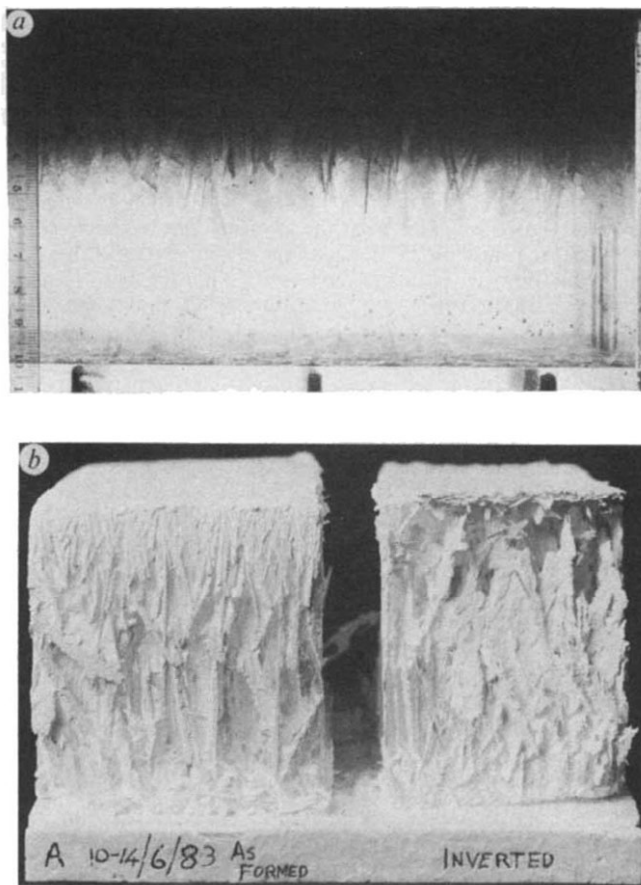


Fig. 3 Photographs of an experiment in which aqueous Na_2CO_3 was cooled from above. a, Photograph with back-lighting after 1 h 38 min. Note the growth of long crystals with nearly vertical orientation, followed by a layer of eutectic with a sharp horizontal boundary. The scale on the left indicates the distance (in cm) from the cooled roof. b, The subsequent crystal block after ice was allowed to melt and run out of the upright (left) and inverted (right) halves of the block.

the crust is broken up by the turbulent flow beneath it and falls into the flow, a new crust forms fairly rapidly. Thus a steady-state crust caps the flow during almost all the emplacement period.

Post-emplacement processes

Once the supply of magma has ceased, the komatiite can be expected to drain into any available depressions, which would include the channel incised by thermal erosion. In many lavas only part of the cooling will have been accomplished during active flow and the ponded lava will continue to cool and crystallize. If the crust is thin, theoretical calculations (which will be presented in detail elsewhere) show that the ponded komatiite will continue to cool by thermal convection. By balancing the conductive heat flux across the crust with the convective flux in the interior, we obtain initial cooling rates of tens to hundreds of °C per hour. As the crust thickens from being a few centimetres thick, the lava temperature decreases and eventually turbulent convection will decrease in vigour. At this stage the heat budget is dominated by a balance between the conductive flux across the crust and latent heat release. We envisage that granular olivines suspended in the lava will now fall out to form the peridotitic cumulate layer, typical of the lower parts of many komatiites¹⁰.

In order to simulate the development of spinifex texture, we crystallized saturated aqueous solutions by cooling from above. Figure 3 shows the results of cooling an aqueous solution of

12.3% anhydrous Na_2CO_3 in a cubical container initially at 31 °C by circulating alcohol at -30 °C through the top plate. Initially, vigorous thermal convection occurred and small dendritic crystals of hydrated Na_2CO_3 nucleated on the roof. At first the crystals showed a wide range of orientations with respect to the roof (Fig. 3a). As the experiment progressed, however, the crystals increasingly became oriented perpendicular to the roof (Fig. 3b). At the same time, a stagnant pool of less dense, residual fluid formed between the growing crystals due to compositional convection¹⁹. The tips of the crystals extended beyond this layer into the saturated, convecting fluid beneath. Experiments on other saturated solutions of Na_2SO_4 and KNO_3 mirrored these features, confirming that the general behaviour is not dependent on composition.

Geological interpretations

Our analysis shows that komatiites can become significantly contaminated by assimilation of the underlying rock (Fig. 2C). We emphasize three points concerning the contamination process. First, floor rocks will be quickly melted and efficiently mixed into the turbulent flow. Xenoliths would not be expected to survive. Second, the chemical signature of contamination would not be extreme; only elements strongly concentrated in the contaminant and depleted in the komatiite would be noticeably affected, and the evidence for this would be most apparent in measurements of the ratios of elements with different compatibilities. Third, the amount of contamination is strongly dependent on flow conditions and distance downstream. As most eruptions probably fluctuate in intensity, the amount of contamination is likely to be very variable even within one flow. Non-systematic variations with indices of olivine fractionation would be anticipated for those elements that are sensitive indicators of contamination.

An application of the thermal erosion model relates to the origin of nickel sulphide mineralization, which we suggest could have been formed by komatiites melting underlying sulphur-rich sediments. Contamination of 10% of a sediment containing 6% sulphur could increase the sulphur content of the lava by 0.6%. The sulphur forms an immiscible sulphide liquid which extracts nickel from the komatiite and segregates to the base of the flow⁵. The sulphide liquid could separate effectively even from the turbulently flowing lava because of its high density ($\sim 4,500 \text{ kg m}^{-3}$), especially if it coagulated to form large drops with high settling velocities.

At Kambalda in Western Australia, the nickel sulphide ores are found at the base of komatiite flows where the lavas are thickest⁹. The long linear depressions which the ores occupy have been interpreted as pre-existing features down which the lavas were channelled. However, it would then be expected that sediment would accumulate in the channels and be thickest in these areas, whereas sediment rarely underlies the thickest komatiite flows and nickel sulphide is present there instead²⁰.

This antithetic relationship between the occurrence of sediment and ore is consistent with the suggestion that the

depressions are thermal erosion channels where the lava has digested the sediment and cut into underlying basalt. The model also avoids the problem of trying to explain why highly magnesian melts carry such high contents of sulphur²⁰. Thus, this model explains many of the features of mineralization, such as the regional association of sediment and ore-bearing komatiites, evident throughout the Western Australia nickel province. It remains to be seen, however, whether the model is of more general applicability.

Our work also relates to the origin of the characteristic textures and layering of komatiite flows²¹. Skeletal olivine morphologies and mineralogical peculiarities, such as crystallization of clinopyroxene, appear to demand either high rates of cooling or melts undercooled by hundreds of °C^{17,18,22}. Cooling rates, based on conductive models²³, are orders of magnitude less than the rates required by experimental studies of olivine growth^{17,18}. Thus, it is difficult to see how the slow cooling rates characteristic of conductive models can cause these textures. Our analysis of the post-emplacement phase, combined with Donaldson's experimental results¹⁷, indicates that rapid cooling rates and large undercoolings can be achieved. Our experiments also indicate that compositional convection can significantly influence the morphology of crystals growing from the roof. Skeletal and dendritic crystal morphologies and metastable crystallization could be a consequence of both thermal and compositional convection.

Our study suggests that during flow, high cooling rates cause either pre-existing phenocrysts to grow, or high nucleation rates. Granular equant olivines remain suspended during turbulent flow and initially on emplacement, when thermal convection might occur. As convective stirring diminishes in vigour, the olivines settle out to form a cumulate layer. Olivine crystals nucleate on the roof and at first grow as dendrites in many orientations. However, compositional convection causes a pool of differentiated melt to form between the crystals beneath the roof. As the pool develops, the tips of olivine crystals grow preferentially at the margins, as in the experiment, and the olivines increasingly grow perpendicular to the roof.

We conclude that komatiites probably flowed turbulently and cooled in part by convection. One consequence of this behaviour is that komatiites can melt and assimilate underlying rock to form deep thermal erosion channels. Assimilation of sulphur-rich sediments may result in the formation of nickel ore deposits. After emplacement, komatiites can continue to cool by thermal convection. Nucleation of crystals on the roof results in the development of spinifex texture. All these processes have important influences on the development of the characteristic geology, textures, geochemistry and mineralization of these rocks.

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1. Viljoen, M. J. & Viljoen, R. P. *Spec. Publ. geol. Soc. S. Afr.* **2**, 87-112 (1969).
2. Arndt, N. T. & Nisbet, E. G. (eds) in *Komatiites* Ch. 2, 19-26 (Allen & Unwin, London, 1982).
3. Bickle, M. J. in *Komatiites* (eds Arndt, N. T. & Nisbet, E. G.) Ch. 27, 479-494 (Allen & Unwin, London, 1982).
4. Donaldson, C. H. in *Komatiites* (eds Arndt, N. T. & Nisbet, E. G.) Ch. 16, 213-244 (Allen & Unwin, London, 1982).
5. Naldrett, A. J. *Econ. Geol.* **74**, 1520-1528 (1979).
6. Elder, J. W. *Geothermal Systems* (Academic, New York, 1982).
7. Wilson, L. & Head, J. W. *J. geophys. Res.* **86**, 2971-3001 (1981).
8. Nisbet, E. G. & Walker, D. *Earth planet. Sci. Lett.* **60**, 103-113 (1982).
9. Leshner, C. M., Lee, R. F., Groves, D. I., Bickle, M. J. & Donaldson, M. J. *Econ. Geol.* **76**, 1714-1728 (1981).
10. Arndt, N. T., Naldrett, A. J. & Pyke, D. R. *J. Petrol.* **18**, 319-369 (1977).
11. Hulme, G. *Modern Geol.* **4**, 107-117 (1973).
12. Hulme, G. *Geophys. Surv.* **5**, 245-279 (1982).
13. Britter, R. E. & Simpson, J. E. *J. Fluid Mech.* **88**, 223-240 (1978).
14. Simpson, J. E. & Britter, R. E. *J. Fluid Mech.* **94**, 477-495 (1979).
15. Holman, J. P. *Heat Transfer* (McGraw Hill, New York, 1976).
16. Bickle, M. J. *Prog. exp. Petrol.* **4**, 187-195 (1978).
17. Donaldson, C. H. *Contr. Miner. Petrol.* **57**, 187-213 (1976).
18. Donaldson, C. H. *Contr. Miner. Petrol.* **69**, 21-32 (1979).
19. Chen, C. F. & Turner, J. S. *J. geophys. Res.* **85**, 2573-2593 (1980).
20. Gresham, J. J. & Loftus-Hills, G. D. *Econ. Geol.* **76**, 1373-1416 (1981).
21. Pyke, D. R., Naldrett, A. J. & Eckstrand, O. R. *Bull. geol. Soc. Am.* **84**, 955-978 (1973).
22. Campbell, I. H. & Arndt, N. T. *Geol. Mag.* **119**, 605-610 (1982).
23. Usselman, T. M., Hodge, D. S., Naldrett, A. J. & Campbell, I. H. *Can. Miner.* **17**, 361-372 (1979).

3.2 Å structure of the copper-containing, oxygen-carrying protein *Panulirus interruptus* haemocyanin

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*For the first time, the three-dimensional structure of a member of the copper-containing class of oxygen-carrying proteins—the haemocyanins—has been determined. The structure of *Panulirus interruptus* haemocyanin at 3.2 Å resolution shows that each subunit of the 450,000-molecular weight hexamer is folded into three domains, and that the binuclear copper site is situated in the centre of the second domain. Amino acid sequence comparisons suggest that the polypeptide fold of *P. interruptus* haemocyanin is common to all arthropod haemocyanins.*

OXYGEN is transported in living organisms by three completely different classes of proteins: haemoglobins, haemerythrins and haemocyanins. Several three-dimensional structures, and numerous amino acid sequences are known for haemoglobins and haemerythrins^{1–7}. Haemocyanins have, so far, been much less well characterized at the atomic level. However, this picture is altering rapidly as recently a wealth of haemocyanin sequence information has become available^{8–19}. Here the folding of the polypeptide chain of a haemocyanin molecule is presented for the first time.

Haemocyanins are large, non-haem, copper-containing proteins which occur freely dissolved in the haemolymph of many invertebrate species. Two major classes exist: molluscan and arthropodan haemocyanins, which have completely different molecular architectures. Arthropodan haemocyanins consist of hexamers, or multi-hexamers, with subunits of molecular weight (MW) ~75,000, while molluscan haemocyanins are cylindrical oligomers with subunits of molecular weight ~400,000 (refs 20–25).

The oxygen-binding centres of haemocyanins each contain a pair of copper ions and have been the subject of extensive spectroscopic analysis. It is generally believed that the oxygen-binding centres of arthropodan and molluscan haemocyanins are quite similar^{25–27}. Furthermore, the spectrum of the binuclear copper-containing oxytyrosinase is remarkably similar to that of oxyhaemocyanin^{28–30}. Other proteins are known also to contain binuclear copper sites, such as ascorbate oxidase, laccase and ceruloplasmin^{31–33}, so knowledge of the binuclear copper site in arthropodan haemocyanins may lead to a better understanding not only of these sites in molluscan haemocyanins but also of those in several enzymes.

We have investigated the haemocyanin of the spiny lobster *Panulirus interruptus*, an arthropod. It is one of the smallest haemocyanins known, but still has a molecular weight of ~450,000 (ref. 34). It is a hexamer, each subunit containing one copper pair required for its oxygen transport function^{25,35}. The oxygen-binding properties of *P. interruptus* haemocyanin^{34–37} exhibit a distinct cooperativity, as do those of all haemocyanins²⁵.

As is common among arthropodan haemocyanins²⁵, *P. interruptus* haemocyanin displays subunit heterogeneity¹³. At least three types of subunits occur, designated a, b and c (formerly called '94K', '90K' and '80K', see below). The crystals used for the X-ray studies were grown from native *P. interruptus* haemocyanin. When the crystals were dissolved it was found that they only contain a and b subunits, in roughly equal amounts (J. P. van Eerd, unpublished results). Although immunological data suggest that these two types of subunits differ in amino acid sequence by ~18% (ref. 38), their N-terminal sequences

are very similar¹³ and no problems were encountered in interpreting the electron density distribution. The polypeptide chain could be followed very easily in a 3.2 Å electron density map of a molecule with a molecular weight of nearly half a million even though for half of the reflections no heavy atom phase information was available.

Amino acid sequence

The amino acid sequence determination was carried out on subunit a of *P. interruptus* haemocyanin. This subunit was formerly called '94K' after its apparent molecular weight (94,000) on SDS-polyacrylamide gels¹³. This nomenclature has now been abandoned, because, as shown below, the number of residues appears to be ~660, indicating a subunit molecular weight of ~75,000. This agrees with earlier gel filtration and sedimentation equilibrium studies by Kuiper *et al.*³⁴.

As a starting point for the primary structure determination, limited proteolysis of subunit a was carried out¹⁴. This generated an 18K fragment, derived from the N-terminus of the subunit, a 71K fragment from the C-terminus and a glycopeptide from in between these two fragments. The apparent molecular weights of these fragments were determined from their mobilities on SDS-polyacrylamide gels. For the N-terminal fragment the molecular weight determined agrees well with its length of 160 residues. The 71K fragment, however, has only ~485 residues, and its anomalous behaviour on SDS gels is not understood.

The tryptic fragments were subjected to further digestions, followed by amino acid sequence analysis. Parts of the amino acid sequence of *P. interruptus* haemocyanin, including previously published sequences^{13–17}, are presented in Fig. 1.

Crystal structure determination

The monoclinic crystals, studied at pH 4.5, contained the complete 450,000-MW hexamer in the asymmetrical unit^{34,39}. The crystal structure of *P. interruptus* haemocyanin at 5.0 Å has been reported elsewhere³⁹. These studies revealed the shape and the 32 point group symmetry of the hexamer, the orientation of the molecular symmetry axes and the molecular centre. No heavy-atom derivative information was used beyond 4 Å. The procedure for obtaining the ultimate 3.2-Å electron density map was as follows.

(1) The 36 heavy-atom sites of the K₂PtCl₄ derivative and the 70 *o*-mercury phenol positions known from the 5-Å studies were optimized, by the lack of closure-error refinement method⁴⁰, against the 4-Å native and derivative data sets, followed by phase⁴¹ and electron density calculations.

(2) The resultant 4.0-Å multi-isomorphous replacement electron density map was subjected to six cycles of Brice's molecular replacement program for phase improvement⁴². The

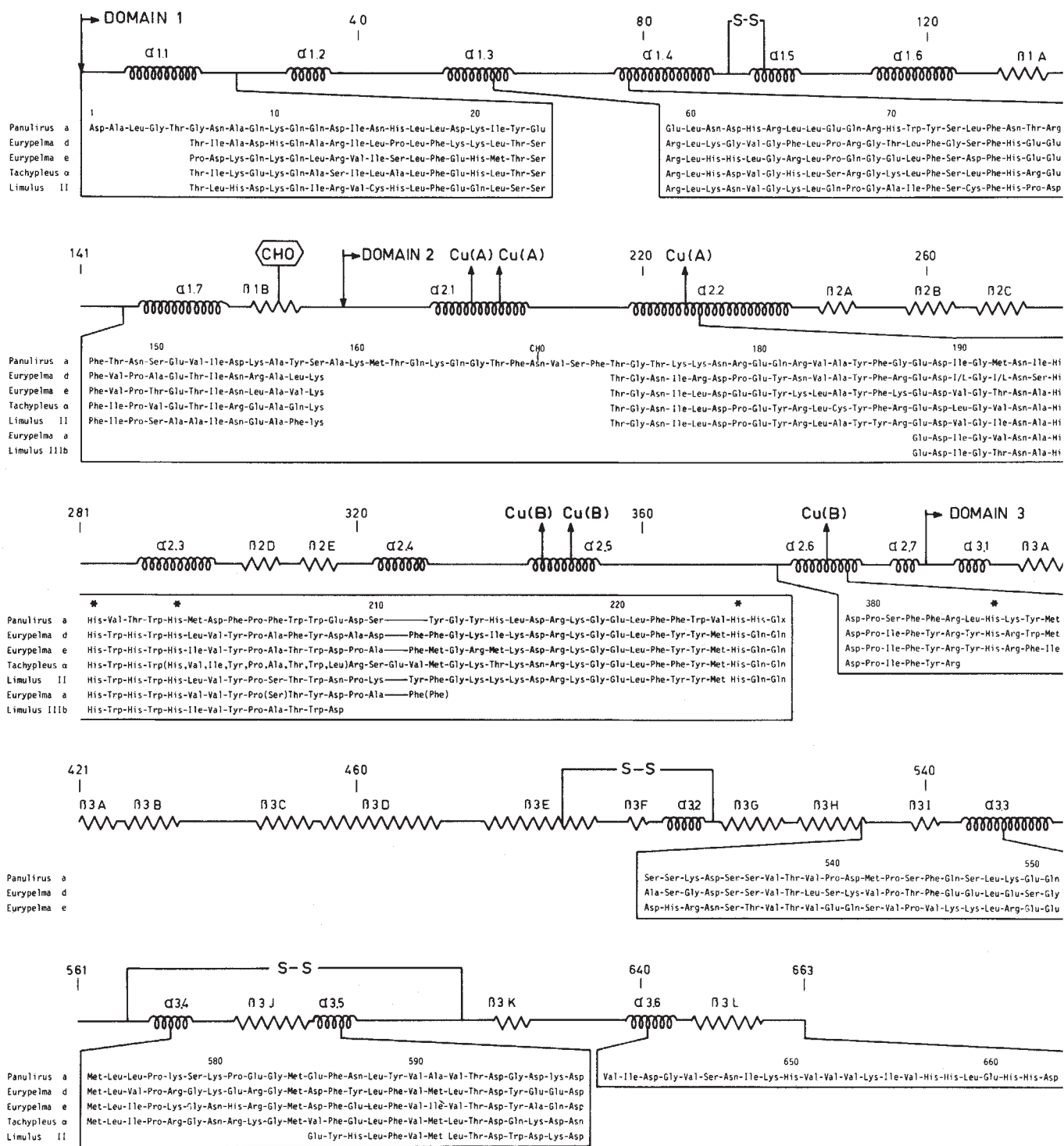


Fig. 1 Secondary structure and partial amino acid sequence of *Panulirus interruptus* haemocyanin subunit a, together with sequence information for several other arthropodan haemocyanins. The carbohydrate moiety (CHO), as well as the copper ligands (vertical arrows and asterisks) are indicated. The starting points of the different domains are indicated by the horizontal arrows. The α -helices are indicated by the coiled line, and the β -strands by the zig-zag. A two-character identifier is assigned to each α -helix and β -strand: the first character is the domain number, the second gives the position of a secondary structure element within its domain (for example, $\alpha 3.4$ is the fourth helix in the third domain; $\beta 2C$ is the third β -strand in the second domain). The amino acid numbering scheme must be considered as tentative, as the *P. interruptus* sequence is not yet complete and the number of residues cannot be established with certainty from the X-ray analysis at the present resolution. For the regions shown, the *P. interruptus* sequence data fit well into the electron density map. The disulphide bridges, indicated by S-S, are based on the electron density map, supported by the sequence information that residue 92 is a cysteine. The first and last three residues of the polypeptide chain are invisible in the map. Weak, but continuous, density occurs for residues 562–566 and 603–613. These weaker densities may be due to different courses of the polypeptide chain in the a and b subunit. Otherwise the entire polypeptide chain is well defined, including numerous side chains. As the N-terminal as well as the C-terminal fragments, of which the amino acid sequences are shown, could be unambiguously placed in the electron density map, as shown, there is no doubt that the complete polypeptide chain has been traced. For selected regions of the chain, apparently related amino acid sequences of other arthropodan haemocyanins are presented. The sequences shown are: *Eurytelma californicum* subunits d (ref. 12), e (ref. 11) and a (ref. 19); *Tachyplesus tridentatus* chain α (ref. 8); and *Limulus polyphemus* subunits II (refs 9 and 10, and E. Yokota and A. F. Riggs, personal communication) and IIIb (ref. 19).

shifts in the phases (Table 1) and the improvement in the electron density map were considerable. The copper positions became evident at this stage⁴³.

(3) The phases obtained from Fourier inversion of the averaged electron density map were added to the known phases in thin shells of increasing resolution, containing 1,900–3,300 reflections. After one phase extension step, three cycles of density averaging and phase improvement were performed at constant resolution. Next, a new shell of reflections with unknown phases was added, and the procedure repeated. After the tenth and last extension step, 11 phase improvement cycles were performed.

The 3.2-Å data set contained 63,842 amplitudes, of which 31,121 had no heavy-atom derivative phase information. The statistics of the procedure are given in Table 1.

Polypeptide chain folding

The resultant 3.2-Å averaged electron density map proved to be of excellent quality (Fig. 2). It appeared possible to trace the complete polypeptide chain, to indicate disulphide bridges and to position several peptides for which the amino acid sequence was known (Fig. 1). The copper ions were by far the most prominent features of the map. The carbohydrate moiety^{14,44} was clearly visible, as shown in Fig. 2.

A *P. interruptus* haemocyanin subunit folds its ~660 residues into an irregular ellipsoid of dimensions 48×55×80 Å, in accordance with electron microscopic data^{20,22}. There are three distinct domains (Figs 3, 4). The first domain comprises 177 residues and is virtually all helical (Figs 1, 3d, 4a). A disulphide bridge occurs between residues 92 and 97. The last β-strand of the first domain is incorporated into a small, three-stranded β-structure together with two strands provided by one of the long loops of the third domain (Fig. 3c, d). This β-strand of domain 1 contains the single carbohydrate moiety of *P. interruptus* haemocyanin, which is covalently attached to residue Asn

Table 1 3.2-Å phase statistics

Resolution limits (Å)	No. of reflections	R-factor* (%)	Figure-of-merit†
20.0–8.94	3,261	18.5	0.75
8.94–6.32	6,180	15.4	0.82
6.32–5.16	7,646	18.3	0.81
5.16–4.47	9,004	20.6	0.78
4.47–4.00	9,696	23.6	0.76
4.00–3.65	10,113	26.7	0.74
3.65–3.38	9,960	30.1	0.71
3.38–3.16	7,982	35.7	0.65
Total	63,842	23.5	0.75

For the double isomorphous replacement phases, out to 4 Å, the average figure-of-merit (as defined by Blow and Crick⁴¹) was 0.57 for 32,721 reflections. After six cycles of phase improvement by density averaging⁴², the average figure-of-merit, as defined by Sim⁶⁶, was 0.76 with an average phase change of 60.4° with respect to the isomorphous replacement phases. The table provides statistics as a function of resolution after the phase extension (as described in the text) out to 3.2 Å. Molecular replacement programs were provided by Dr G. Bricogne and rewritten in FORTRAN 77 for the Cyber 170/760 of the Computer Centre of the University of Groningen, where all computations were performed.

$$R = 100 \times \frac{\sum (|F_{P,calc}| - |F_{P,obs}|)}{\sum |F_{P,calc}|}$$

with

$$|F_{P,calc}| = k \exp \left[-B \left(\frac{\sin \theta}{\lambda} \right)^2 \right] |F_{P,inv}|$$

where $|F_{P,inv}|$ is the structure amplitude obtained by Fourier inversion of the averaged electron density map; k and B are relative scaling and temperature factors obtained by a relative Wilson plot.

† The average phase difference between the phases after six cycles of molecular replacement at 4 Å resolution and the phases obtained by the multiple isomorphous replacement method.

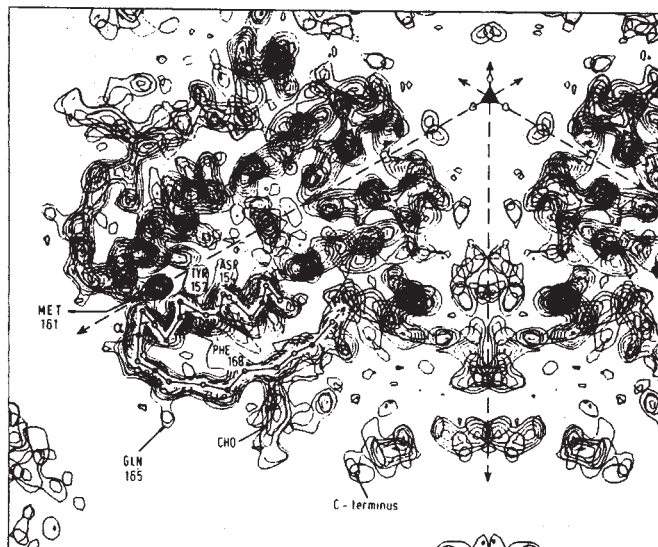


Fig. 2 Part of the ultimate, averaged, 3.2-Å electron density map. Five sections are shown through the centre of the hexamer, viewed along the local 3-fold axis of symmetry (Δ). The thickness of the slab shown is 6 Å. As the hexamer obeys 32 point group symmetry³⁹, three local 2-fold symmetry axes occur in the central section, as indicated. For one subunit α-carbon positions in α-helix 1.7 and β-strand 1 B are indicated. CHO, the carbohydrate moiety. Some well defined side chains are labelled, and the same densities can be seen in the symmetry-related subunit. The course of the polypeptide chain can be followed without any difficulty, and numerous side chains have a well developed density. In the map actually used for chain tracing and peptide fitting, twice as many sections were used, at distances of 0.75 Å from each other.

169 (ref. 14). It appears that the carbohydrate moiety protrudes into a solvent region and does not, except for the first sugar group, make contacts with amino acid side chains. In this respect, the *P. interruptus* haemocyanin sugar chain is distinctly different from immunoglobulins⁴⁵, but similar to some carbohydrate chains in haemagglutinin⁴⁶ and neuraminidase⁴⁷.

The second domain, comprising residues 177–400, is also largely helical, but has a few β-strands (Figs 1, 3e, 4b). Four of these strands, 2B and 2C, and 2D and 2E, form antiparallel ββ-units. The oxygen-binding site is located in the centre of this globular domain, in between four helices. This site will be described in more detail later.

The third and largest domain consists of residues 400–663. From the central region of this domain extend long loops (Figs 3f, 4c) which appear to function as arms holding the three domains together (Fig. 3c). The central part of this domain is an antiparallel β-barrel consisting of seven strands. The electron density map indicates two disulphide bridges in this domain (Figs 1, 3f, 4c), giving a total of three disulphide bridges in each haemocyanin subunit.

Primary structure comparisons (Fig. 1) show that all three domains contain regions of similar sequence between haemocyanins of different organisms, which suggests strongly that the three-domain structure is a common feature of all arthropodan haemocyanins.

Figure 3a shows a stereoview of the hexamer and Fig. 3b the 'upper' trimer. In the hexamer, all three domains are involved in inter-subunit contacts. For instance, helix 1.7 is in contact with the same helix in a subunit related by a 2-fold axis (see Fig. 2). Helices 2.3 from three subunits interact with each other around the molecular 3-fold axis (Fig. 3b), and helix 3.6 is close to helix 1.3 of another subunit. As in haemoglobin^{1,3} and haemerythrin^{5–7}, the contacts are all mediated by interactions between side chains—no hydrogen bonding interactions between backbone atoms occur.

The oxygen-binding site

The crystals that were used in the X-ray analysis were colourless, which suggests that we have determined the structure of the

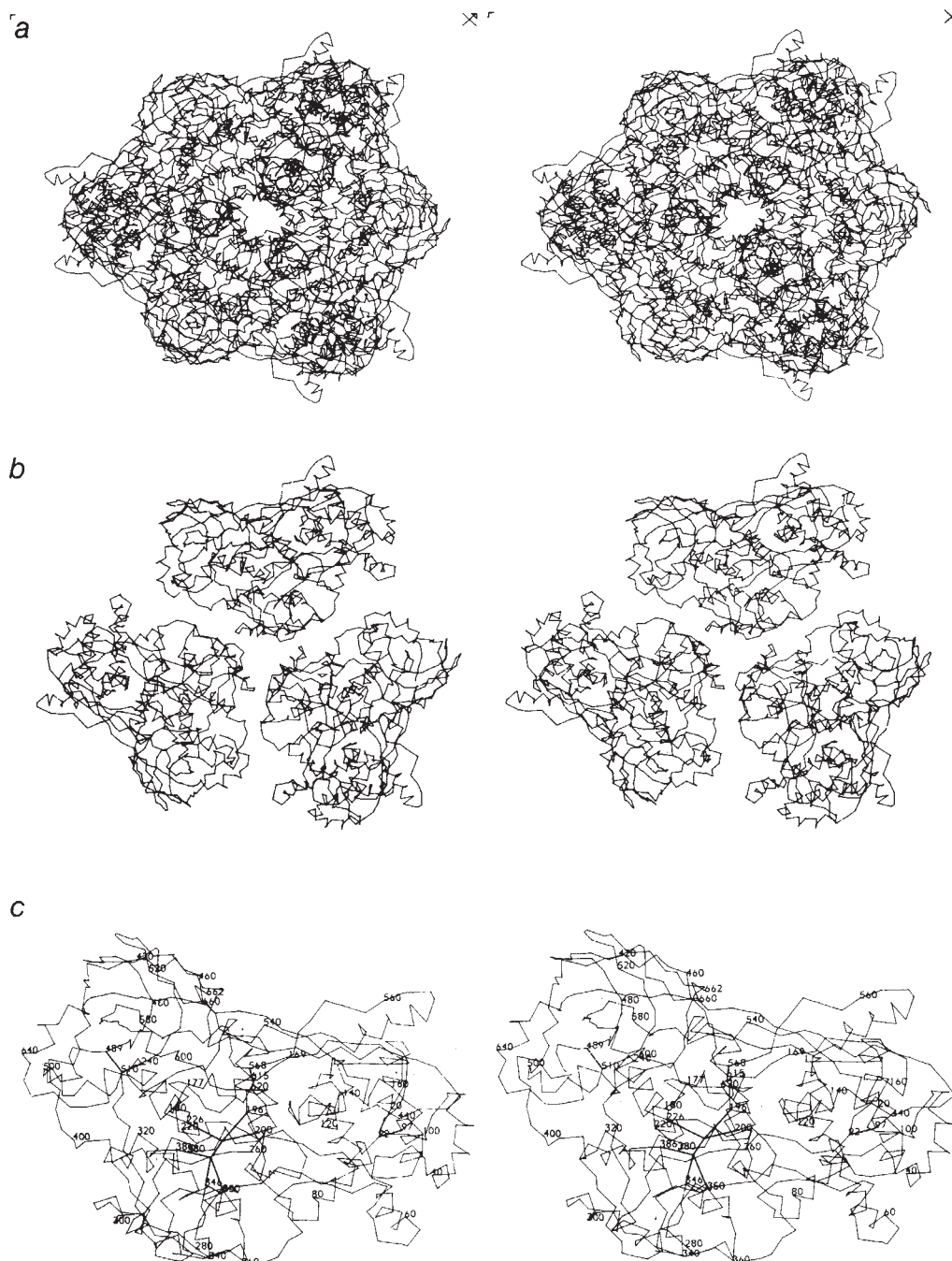


Fig. 3 The three-dimensional structure of *P. interruptus* haemocyanin in stereoscopic projection. Coordinates of the α -carbon, γ -sulphur and copper atoms were directly measured from a 3.2-Å electron density map, which was plotted on transparent sheets at a scale of 1 cm = 4 Å. Although the general course of the chain could be followed without difficulty, α -carbon coordinates of certain loops with less well developed side chains may be less accurate than that of other regions. **a**, The complete hexamer, with a total of 3,978 residues, viewed along the molecular 3-fold axis. The three molecular 2-fold axes of this molecule with point group 32 (ref. 39) are in the plane of the paper. The hexamer can be described as two trimers, related by 2-fold axes and stacked face-to-face upon each other. **b**, A view along the local 3-fold axis of the 'upper' trimer of **a**. **c**, A view along the local 3-fold axis of one subunit only, with the same residues labelled as in **d-f**. Residue 169 contains the carbohydrate moiety. Residue 177 is the end of the first domain, and number 400 the end of the second domain. **d**, The first domain only, in a view which gives a clear picture of the course of the polypeptide chain. The position of the first three residues is arbitrary as no density was visible in the electron density map. One disulphide bridge is indi-

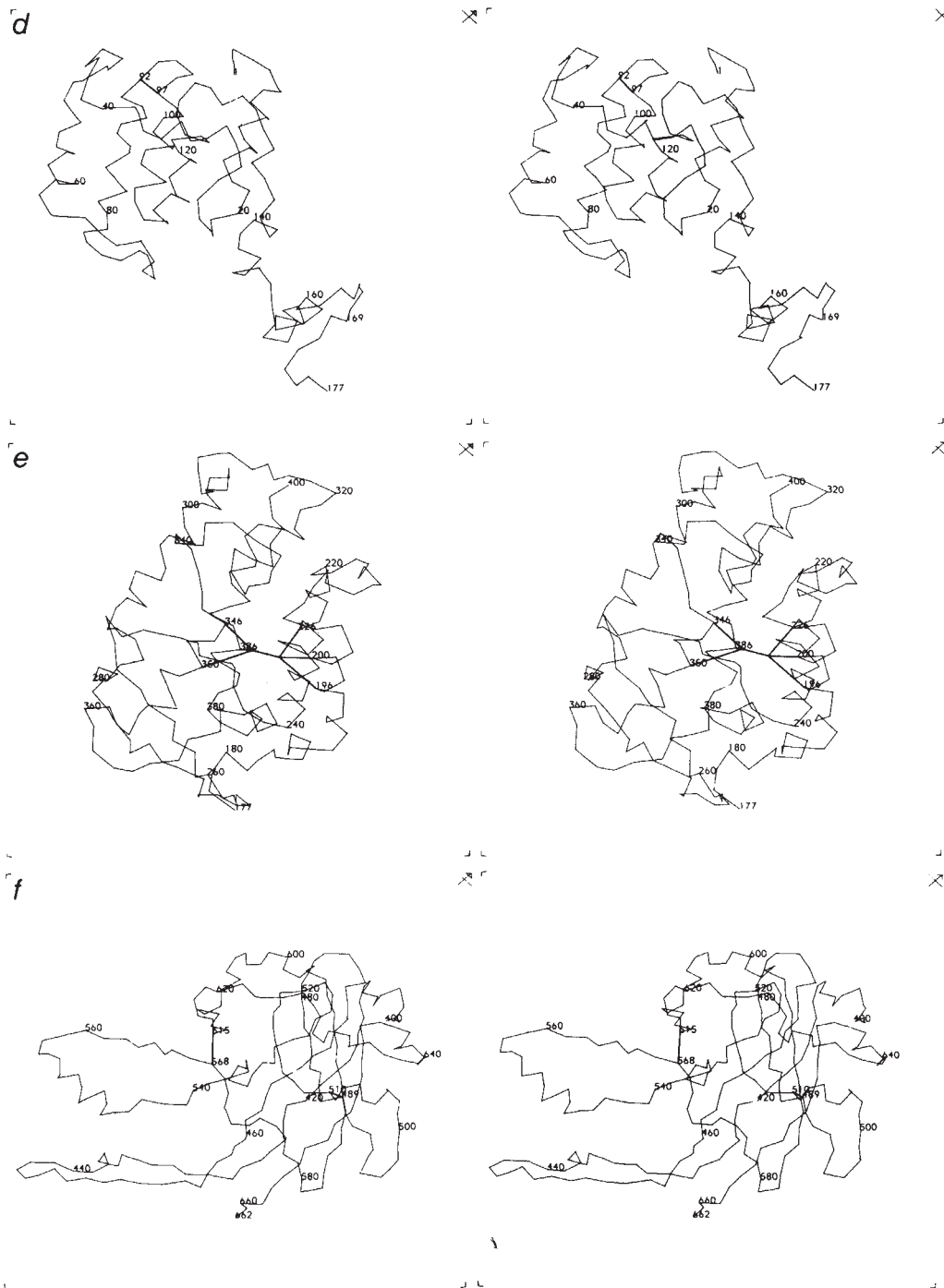
deoxygenated form of haemocyanin. This is consistent with the low oxygen affinity of *P. interruptus* haemocyanin at low pH⁴⁸, and the absence of an absorption band near 340 nm in single-crystal UV spectroscopy (M. Vincent, unpublished data). However, we cannot rule out completely that the structure we have determined is of Met, or half-Met, haemocyanin.

It is clear from the 3.2-Å electron density map that each copper ion has three protein ligands. No evidence for a bridging protein ligand is found. One of the pair of copper ions, Cu(A), is surrounded by residues 196, 200 and 226; the other, Cu(B), is surrounded by residues 346, 350 and 386. Two of the ligands of Cu(A) come from successive turns of helix 2.1, and the equivalent two ligands of Cu(B) come from successive turns of helix 2.5. The third ligand of each copper ion also comes from a helix. The six copper ligands are thus provided by four helices. This is reminiscent of the situation in haemerythrins⁵⁻⁷, but the topologies of the helices surrounding the oxygen-binding sites are different in these two classes of oxygen-binding proteins.

Antiparallel helices both providing ligands for metal ions are also found in Fe-superoxide dismutase^{49,50}.

All six copper ligands in haemocyanin are almost certainly histidines. For four of the ligands (residues 196, 200, 226 and 386) this is clear from the fit of the *Panulirus* haemocyanin amino acid sequence with the electron density map. All other sequenced haemocyanins also have histidine residues at these positions, and very similar sequences in the surrounding regions (Fig. 1). The two remaining Cu(B) ligands have not been sequenced yet for *P. interruptus* haemocyanin. These two ligands must have the sequence L₁-X-X-X-L₂ according to the electron density map, with L₂ approximately 36 residues away from the third ligand. In the sequences of *Tachypleus* haemocyanin⁸, and of two *Eurypelma* haemocyanin subunits^{11,12} the arrangement His-X-X-X-His is observed precisely at the expected position. As the amino acid sequences of these haemocyanins are clearly related to that of *P. interruptus* haemocyanin in other regions (Fig. 1), it is justified to conclude that in arthropodan

cated by a virtual bond between the α -carbon atom of residues 92 and 97. Up to residue 155, this domain is quite globular, but residues 156–177 form an 'appendix' comprising helix 1.7 and β -strand 1 B. Helix 1.7 forms contacts via a 2-fold axis with another subunit as shown in Fig. 2. Strand 1 B contains the carbohydrate chain at position 169, and forms a β -structure with strands 3B and 3C of the long extended loop, 440–480, of the third domain (c). e. The second domain in a view allowing an easy following of the chain. The positions of the copper atoms are indicated and connected to the α -carbon atoms of the liganding residues. This domain is clearly globular and is mainly helical. The six protein ligands of the two copper ions are all histidines (see text). It should be emphasized that, at the present resolution, we cannot exclude the possibility of a small exogenous bridging ligand, such as a μ -oxo atom. f. The third domain oriented in such a manner as to highlight the two long excursions of the chain. Two probable disulphide bridges are indicated. It is quite obvious that this domain is far from globular. The long loops which extend from the central β -barrel make intimate contacts with the two other domains (c). The position of the last three residues is arbitrary as only weak density was present in the electron density map for these residues.



haemocyanins the two copper ions are liganded by six histidines. The involvement of the histidine cluster at position 195–200 in copper binding has been correctly suggested by Schneider *et al.*¹⁹ and Yokata *et al.*⁹. That each copper atom has three ligands agrees well with the suggested model of the oxygen-binding site for deoxyhaemocyanin by Brown *et al.*⁵¹ and Larrabee and Spiro⁵².

The distance between the two electron density maxima representing the copper ions is 3.8 Å, with an error of ~0.4 Å. This corresponds well with the values derived from EXAFS experiments: 3.39 Å⁵¹ for deoxyhaemocyanin; 3.67 Å⁵¹ and 3.55 Å⁵³ for oxyhaemocyanin. The copper-binding centres in the subunits of a haemocyanin trimer (Fig. 3b) are separated by distances of 46 Å. The copper centres in one 'upper' subunit are separated from those in the three 'lower' subunits by distances of 47, 54 and 68 Å. Cooperative interactions between subunits thus must operate over quite large distances in *P. interruptus* haemocyanin.

Binuclear copper sites

No other three-dimensional structures are yet available for proteins with binuclear copper sites. However, the complete amino acid sequence of *Neurospora crassa* tyrosinase⁵⁴, a nearly complete sequence of human ceruloplasmin^{32,33} and small fragments of the sequence of laccase^{55,56}—all of which have binuclear copper sites—are known. Does the sequence His-X-X-His, which provides four of the ligands for each haemocyanin binuclear copper centre, occur in these possibly related proteins?

In tyrosinase this sequence does indeed occur: His 277 and His 281 are separated by three residues and are therefore possible copper ligands. Two other histidine residues in tyrosinase have been implicated by photo-inactivation as copper ligands: His 188 and His 193 (ref. 54). These occur in a sequence resembling that which provides two of the copper ligands in haemocyanin⁹, but they are separated by four, not three, residues. This suggests that there are variations in the structure

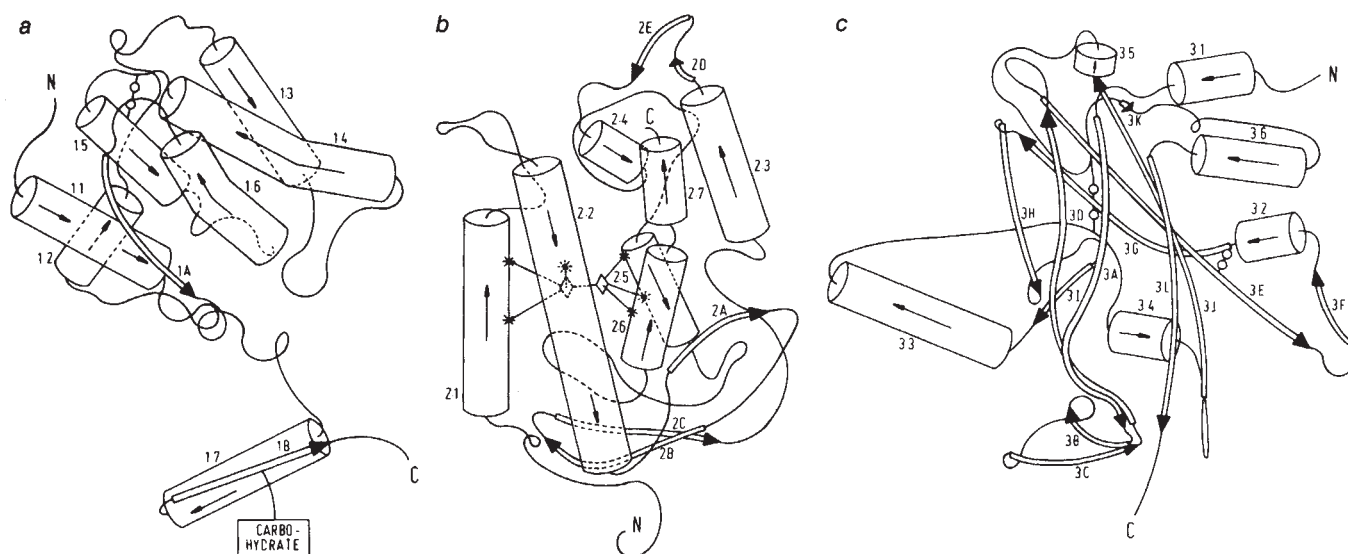
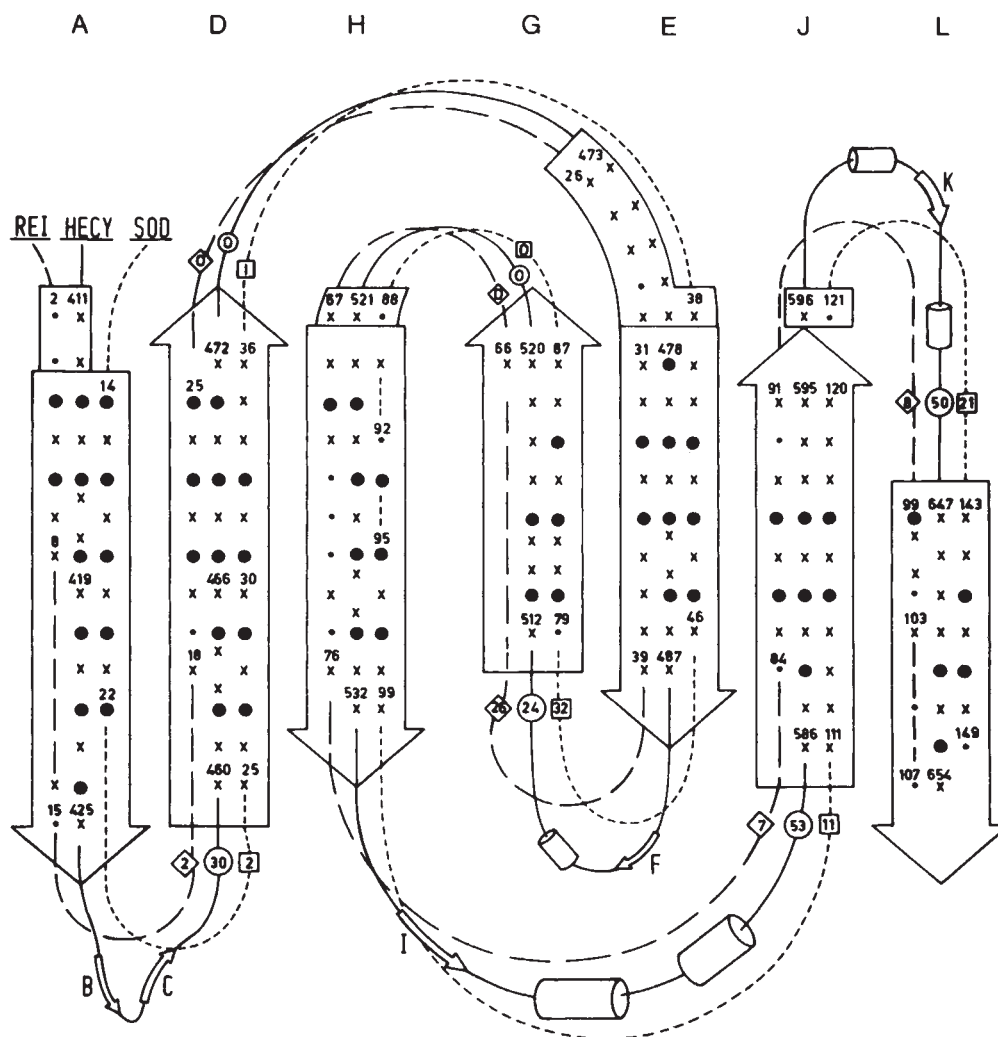


Fig. 4 The three domains of a subunit of *P. interruptus* haemocyanin depicted schematically, viewed from the same direction. **a**, Domain 1: Helices are indicated by cylinders and β -strands by arrows. Secondary structure elements are indicated as in Fig. 1. A disulphide bridge is shown as —○—○—. The 'appendix' formed by helix 1.7 and β -strand 1B is evident. This β -strand contains the carbohydrate moiety. **b**, Domain 2: The copper ions, which are located in the centre of this domain, are indicated by diamonds. All six ligands of the copper ions can clearly be seen to be provided by residues belonging to α -helices. It may be noted that the first ligand of Cu(B), residue 346 of helix 2.5, is situated at the very N-terminal turn of this helix. This is quite different from the first ligand of Cu(A), which resides in the middle of helix 2.1, and from the Fe-ligands in haemerythrin, which are also provided by residues from central parts of helices. **c**, Domain 3: Two probable disulphide bridges are indicated by —○—○—. In this view, one long loop, comprising α 3.3, extends to the left while another one, connecting β -strands 3B and 3C, extends to the back of this picture. (Drawings by A. Volbeda.)

Fig. 5 Alignment of the equivalent amino acid residues in the β -barrels of Cu, Zn superoxide dismutase (SOD)⁶², immunoglobulin REI (REI)⁶³ and the third domain of *P. interruptus* haemocyanin (HECY). The large arrows represent the seven β -strands which form the major part of the barrel. Equivalent α -carbon atoms which superimpose within 3.2 Å with haemocyanin are shown as large dots (●) and crosses (×). Equivalent α -carbon atoms deviating more than 3.2 Å but less than 4.0 Å are indicated by small dots (•). ● Indicate residues that point their side chains into the interior of the β -barrel. Residues marked by • or × do not point towards the interior. Strands G, E, J and L (haemocyanin numbering as in Figs 1 and 4) form one side of the barrel, strands A, D and H the other side. The lengths of the loops connecting the strands are also indicated: those of Cu, Zn superoxide dismutase in squares; those of immunoglobulin REI in circles; and those of haemocyanin in diamonds. (Drawing by A. Volbeda.) It appears that the third domain of haemocyanin has by far the largest loops. The loop connecting strands D and E is very similar in immunoglobulin REI and haemocyanin. The short loop connecting strands G and H is the same in all three cases. The three β -structures appear to resemble each other remarkably well (see text). For Cu, Zn superoxide dismutase and immunoglobulin REI, the coordinate sets 2SOD and 1REI from the Protein Data Bank⁶⁷ have been used for the superposition studies.



of the polypeptide chains surrounding these binuclear copper sites in different molecules, although spectroscopic data suggest that the copper environments are actually very similar²⁸⁻³⁰.

In ceruloplasmin two pairs of histidines separated by three residues occur^{32,33}, and in the short sequence known of laccase⁵⁵ one such pair has been found. On the basis of the haemocyanin structure, these histidines may be binuclear copper ligands, although for ceruloplasmin other models have been suggested^{32,33}.

Molluscan haemocyanin structure

Knowledge of the oxygen-binding domain of arthropodan haemocyanins suggests a model for the molluscan haemocyanin structure. The subunit molecular weight of molluscan haemocyanins is ~400,000, with ~50,000 per binuclear copper site^{20-22,25}. The arthropodan oxygen-binding domain, with ~223 residues, has a molecular weight of ~25,000. Spectroscopic evidence suggests that the oxygen-binding centres of molluscan and arthropodan haemocyanins are very similar²⁵⁻²⁷. The basic 50,000-MW unit in molluscan haemocyanins may therefore consist of an oxygen-binding domain similar to the one in *P. interruptus* haemocyanin, and a second domain which might correspond to the first or third *P. interruptus* haemocyanin domain. The two domains would be in quite intimate contact, forming a globular unit, while an array of eight such units makes up a complete molluscan haemocyanin subunit⁵⁷⁻⁵⁹. Similar relationships between the two haemocyanin classes have been suggested, on different grounds, by Van Holde⁶⁰.

Convergent evolution

The core of the third haemocyanin domain consists of a seven-stranded, antiparallel β -barrel. Surprisingly, the topology of this core is identical to that of the β -barrels in immunoglobulins and in Cu, Zn superoxide dismutase (Fig. 5)⁶¹⁻⁶³. After optimal superposition of ~60 equivalent C α -positions, the three barrels deviate mutually by less than 2.5 Å (r.m.s.).

However, when the available amino acid sequence for the

β -barrel region of *P. interruptus* haemocyanin is compared with the sequences of the other β -barrels, hardly any similarities are observed. This is also the case when the β -barrel sequences of immunoglobulins and Cu, Zn superoxide dismutase are compared⁶¹. This β -barrel structure is probably an inherently stable structure, which allows great variability in length and folding patterns of the loops connecting the strands. The appearance of such β -barrels in domains with totally different functions suggests that the structural relationship is the result of convergent evolution.

Conclusion

The combination of X-ray diffraction and amino acid sequencing techniques described here has allowed the elucidation of the architecture of *P. interruptus* haemocyanin, which is probably common to all arthropodan haemocyanins. Furthermore, a detailed view has been obtained of the oxygen-binding site, which may share many features with the binuclear copper sites found in molluscan haemocyanins and several other proteins. Important questions remain to be answered, however, such as the nature of the contacts between the subunits of this hexameric protein, the structural changes that occur on oxygenation (fortunately, the X-ray analysis of an oxyhaemocyanin is under way^{64,65}), and the relationship between the structures of arthropodan and molluscan haemocyanins. Once these questions are settled, then a complete picture will have been obtained of the three, or perhaps four, solutions nature has found for reversible oxygen transport in living organisms.

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- Perutz, M. F., Muirhead, H., Cox, J. M. & Goaman, L. C. *Nature* **219**, 131-139 (1968).
- Steigemann, W. & Weber, E. *J. molec. Biol.* **127**, 309-338 (1979).
- Dickerson, R. E. & Geis, I. *Hemoglobin: Structure, Function, Evolution and Pathology* (Cummings, Menlo Park, 1983).
- Garlick, R. L. & Riggs, A. F. *J. biol. Chem.* **257**, 9005-9015 (1982).
- Hendrickson, W. A. & Ward, K. B. *J. biol. Chem.* **252**, 3012-3018 (1977).
- Stenkamp, R. E., Sieker, L. C., Jensen, L. H. & McQueen, J. E. *Biochemistry* **17**, 2499-2504 (1978).
- Smith, J. L., Hendrickson, W. A. & Addison, A. W. *Nature* **303**, 86-88 (1983).
- Nemoto, T. & Takagi, T. *Life Chem. Rep.* **1**, Suppl. 1, 89-92 (1983).
- Yokota, E., Moore, M. D., Behrens, P. Q. & Riggs, A. F. *Life Chem. Rep.* **1**, Suppl. 1, 75-80 (1983).
- Moore, M. D. & Riggs, A. F. *Life Chem. Rep.* **1**, Suppl. 1, 93-97 (1983).
- Schneider, H. J. et al. *Hoppe-Seyler's Z. physiol. Chem.* **364**, 1357-1381 (1983).
- Schartau, W. et al. *Hoppe-Seyler's Z. physiol. Chem.* **364**, 1383-1409 (1983).
- Van Eerd, J. P. & Folkerts, A. in *Invertebrate Oxygen-Binding Proteins* (eds Lamy, J. & Lamy, J.) 139-149 (Marcel Dekker, New York, 1981).
- Vereijken, J. M., Schwander, E. H., Soeter, N. M. & Beintema, J. J. *Eur. J. Biochem.* **123**, 283-289 (1982).
- Vereijken, J. M. *Life Chem. Rep.* **1**, Suppl. 1, 99-100 (1983).
- Soeter, N. M. & Beintema, J. J. *Life Chem. Rep.* **1**, Suppl. 1, 101-102 (1983).
- Schwander, E. H. & Vereijken, J. M. *Life Chem. Rep.* **1**, Suppl. 1, 103-106 (1983).
- Jollès, J., Jollès, P., Lamy, J. & Lamy, J. *FEBS Lett.* **106**, 289-291 (1979).
- Schneider, H. J. et al. *Hoppe-Seyler's Z. physiol. Chem.* **363**, 487-492 (1982).
- Fernández-Morán, H., Van Bruggen, E. F. J. & Ohtsuki, M. *J. molec. Biol.* **16**, 191-207 (1966).
- Mellema, J. E. & Klug, A. *Nature* **239**, 146-150 (1972).
- Van Bruggen, E. F. J. et al. in *Electron Microscopy of Proteins* Vol. 1 (ed. Harris, M.) 1-38 (Academic, New York, 1981).
- Lamy, J., Bijlholt, M. M. C., Sizaret, P.-Y., Lamy, J. & van Bruggen, E. F. J. *Biochemistry* **20**, 1849-1856 (1981).
- Bijlholt, M. C., Van Heel, M. G. & Van Bruggen, E. F. J. *J. molec. Biol.* **161**, 139-153 (1982).
- Van Holde, K. E. & Miller, K. I. *Q. Rev. Biophys.* **15**, 1-129 (1982).
- Himmelwright, R. S., Eickman, N. C., LuBien, C. D. & Solomon, E. I. *J. Am. chem. Soc.* **102**, 5378-5388 (1980).
- Torensma, R. & Phillips, J. C. *FEBS Lett.* **130**, 314-316 (1981).
- Schoot Uiterkamp, A. J. M. & Mason, H. S. *Proc. natn. Acad. Sci. U.S.A.* **70**, 993-996 (1973).
- Eickman, N. C., Solomon, E. I., Larrabee, J. A., Spiro, Th. G. & Lerch, K. *J. Am. chem. Soc.* **100**, 6529-6531 (1978).
- Himmelwright, R. S., Eickman, N. C., LuBien, C. D., Lerch, K. & Solomon, E. I. *J. Am. chem. Soc.* **102**, 7339-7344 (1980).
- Urbach, F. L. in *Metal Ions in Biological Systems* Vol. 13 (ed. Sigel, H.) 73-115 (Marcel Dekker, New York, 1981).
- Rydén, L. *Proc. natn. Acad. Sci. U.S.A.* **79**, 6767-6771 (1982).
- Takahashi, N. et al. *Proc. natn. Acad. Sci. U.S.A.* **80**, 115-119 (1983).
- Kuiper, H. A. et al. *J. molec. Biol.* **99**, 619-629 (1975).
- Kuiper, H. A., Antonini, E. & Brunori, M. *J. molec. Biol.* **116**, 569-576 (1977).
- Kuiper, H. A. et al. *Biochemistry* **18**, 5849-5854 (1979).
- Antonini, E., Brunori, M., Colosimo, A., Kuiper, H. A. & Zolla, L. *Biophys. Chem.* **18**, 117-124 (1983).
- Folkerts, A. & Van Eerd, J. P. in *Invertebrate Oxygen-Binding Proteins* (eds Lamy, J. & Lamy, J.) 215-225 (Marcel Dekker, New York, 1981).
- van Schaick, E. J. M., Schutter, W. G., Gaykema, W. P. J., Schepman, A. M. H. & Hol, W. G. J. *J. molec. Biol.* **158**, 457-485 (1982).
- Dickerson, R. E., Weinzierl, J. E. & Palmer, R. A. *Acta crystallogr.* **B24**, 997-1003 (1968).
- Blow, D. M. & Crick, F. H. C. *Acta crystallogr.* **12**, 794-802 (1959).
- Bricogne, G. *Acta crystallogr.* **A32**, 832-847 (1976).
- Gaykema, W. P. J., Van Schaick, E. J. M., Schutter, W. & Hol, W. G. J. *Chemica Scripta* **21**, 19-23 (1983).
- Van den Berg, A. A., Gaastra, W. & Kuiper, H. A. in *Structure and Function of Haemocyanin* (ed. Bannister, J. V.) 6-12 (Springer, Berlin, 1977).
- Deisenhofer, J. *Biochemistry* **20**, 2361-2370 (1981).
- Wilson, I. A., Skehel, J. J. & Wiley, D. C. *Nature* **289**, 366-373 (1981).
- Varghese, J. N., Laver, W. G. & Colman, P. M. *Nature* **303**, 35-40 (1983).
- Kuiper, H. A., Coletta, M., Zolla, L., Chiancone, E. & Brunori, M. *Biochim. biophys. Acta* **626**, 412-416 (1980).
- Ringe, D., Petsko, G. A., Yamakura, F., Suzuki, K. & Ohmori, D. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3879-3883 (1983).
- Stalling, W. C., Powers, T. B., Patridge, K. A., Fee, J. A. & Ludwig, M. L. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3884-3888 (1983).
- Brown, J. M., Powers, L., Kincaid, B., Larrabee, J. A. & Spiro, T. G. *J. Am. chem. Soc.* **102**, 4210-4216 (1980).
- Larrabee, J. A. & Spiro, Th. G. *J. Am. chem. Soc.* **102**, 4217-4223 (1980).
- Co, M. S., Hodgson, K. O., Eccles, Th. K. & Lontie, R. *J. Am. chem. Soc.* **103**, 984-986 (1981).
- Lerch, K. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3635-3639 (1978).
- Briving, C. thesis, Univ. Göteborg (1975).
- Briving, C., Gandvik, E.-K. & Nyman, P. O. *Biochem. biophys. Res. Commun.* **93**, 454-461 (1980).
- Brouwer, M., Wolters, M. & Van Bruggen, E. F. J. *Biochemistry* **15**, 2618-2623 (1976).
- Siezen, R. J. & Van Bruggen, E. F. J. *J. molec. Biol.* **90**, 77-89 (1974).
- Gielens, C., Verschueren, L. J., Préaux, G. & Lontie, R. *Eur. J. Biochem.* **103**, 463-470 (1980).
- Van Holde, K. E. *Life Chem. Rep.* **1**, Suppl. 1, 403-412 (1983).
- Richardson, J. S., Richardson, D. C., Thomas, K. A., Silverton, E. W. & Davies, D. R. *J. molec. Biol.* **102**, 221-225 (1976).
- Tainer, J. A., Getzoff, E. D., Beem, K. M., Richardson, J. S. & Richardson, D. C. *J. molec. Biol.* **160**, 181-217 (1982).
- Epp, O. et al. *Eur. J. Biochem.* **45**, 513-524 (1974).
- Magnus, K. A. & Love, W. E. *J. molec. Biol.* **116**, 171-173 (1977).
- Magnus, K. A. & Love, W. E. *Life Chem. Rep.* **1**, Suppl. 1, 61-64 (1983).
- Sim, G. A. *Acta crystallogr.* **12**, 813-815 (1959).
- Bernstein, F. C. et al. *J. molec. Biol.* **112**, 535-542 (1977).

Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule

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The ability of fibronectin to bind cells can be accounted for by the tetrapeptide L-arginyl-glycyl-L-aspartyl-L-serine, a sequence which is part of the cell attachment domain of fibronectin and present in at least five other proteins. This tetrapeptide may constitute a cellular recognition determinant common to several proteins.

FIBRONECTIN is a well characterized extracellular, structural glycoprotein which interacts strongly with other extracellular matrix molecules and which causes the attachment and spreading of most cells when presented to them as an insoluble substrate. This latter property is of considerable interest because an understanding of how cells interact with their surroundings may lead to an understanding of differentiation and of cell migration, for example, that which occurs during embryonic development or in tumour metastasis.

By using a monoclonal antibody which inhibits cell attachment, we have recently isolated, as a peptic fragment of molecular weight (MW) 11,500 (11.5K), the domain within the fibronectin molecule which endows it with the ability to interact with the cell surface of fibroblasts^{1,2}, and we have determined the complete amino acid sequence of this fragment². This domain contained all of the cell attachment activity to be found among the proteolytic fragments of the fibronectin molecule¹. By using synthetic peptides modelled from the sequence of this domain, we have described a 30-amino acid synthetic peptide which carries the cell attachment-promoting activity³. By using smaller synthetic peptides, we have now localized this function of the fibronectin molecule to a sequence of only four of these amino acids and we have shown that the same tetrapeptide is present in some other proteins which may interact with cells, including fibrinogen⁴, a surface protein of *Escherichia coli*⁵, and a Sindbis virus coat protein⁶.

Cell attachment-promoting activity

The 30-amino acid synthetic peptide described previously placed the cell attachment site within the COOH-terminal quarter of the 108-amino acid cell attachment domain³. At that time, we predicted, by the method of Rose⁷, that the cell attachment-promoting activity of the fibronectin molecule would probably be localized close to a hydrophilic stretch of amino acids near the NH₂-terminus of that peptide. Further analysis of the cell attachment domain, using methods for predicting secondary structure described elsewhere^{8,9}, showed that the group of residues boxed in Fig. 1 may be a β -turn which probably forms a hydrophilic loop at the surface of the molecule and is thus available to interact with the cell surface. To test whether these amino acids were involved in the cell attachment activity of the peptide, we obtained several synthetic peptides (Fig. 2a), coupled them to protein-coated plastic and tested each for cell attachment-promoting activity (Fig. 2b). All the peptides that promoted the attachment of rat kidney fibroblasts (NRK cells) contained the sequence Arg-Gly-Asp-Ser (or Arg-Gly-Asp-Cys) (Fig. 2b). The activity of the peptides decreased somewhat with size, perhaps due to a decrease in the stability of their conformation or to a relative inaccessibility on the substrate. The tetrapeptide, Arg-Gly-Asp-Ser, itself was active when immobilized on Sepharose beads at the end of a C₆ spacing arm (Fig. 3). Poor accessibility due to its small size or low efficiency of the coupling methods used for this peptide may account for

the fact that we could not demonstrate activity in this peptide after coupling to protein-coated plastic.

Analysis of the data in Fig. 2 indicates that the serine residue of the tetrapeptide sequence can be replaced by a chemically similar cysteine residue without substantial loss of the activity (peptide IVA1a in Fig. 2). The activity was lost, however, when the arginine or aspartic acid residues were selectively deleted (Fig. 2) or if the synthetic peptides were cleaved at the arginine with trypsin (data not shown). Also, substitution of the glycine with the bulky valine residue (peptide RVDSPAC in Fig. 2) abolished the activity. Moreover, as can be seen in Fig. 4a, substitution of aspartic acid with asparagine greatly diminished the activity of the resulting tetrapeptide. We conclude from these results and from the data presented below that the Arg-Gly-Asp sequence must be maintained to preserve activity, while some variation at the position occupied by the serine residue is compatible with activity.

Inhibition of cell attachment

We were unable to inhibit the attachment of cells to fibronectin-coated surfaces with soluble fibronectin, nor could we inhibit such attachment with obtainable concentrations of the 30-amino acid, cell attachment-promoting synthetic peptide³. A possible explanation for this lack of inhibition is that the affinity of a

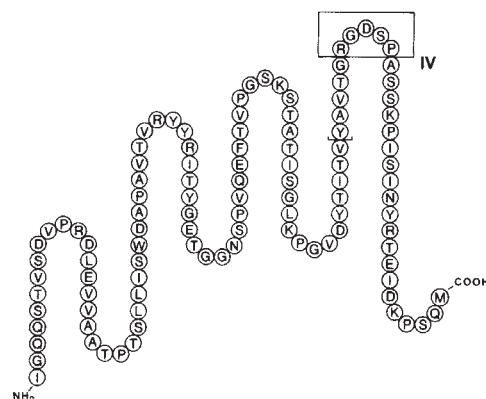


Fig. 1 Prediction of the secondary structure of the 11.5 K cell attachment domain of fibronectin. The methods of Chou and Fassman⁸ and Kyte and Doolittle⁹ were used to predict the probable secondary conformation of the cell attachment domain of fibronectin based solely on the primary structure². The peptide consists of a series of potential β -turns, the most hydrophilic of which (boxed) is shown here to contain the cell attachment signal. Peptide IV, which was shown previously to contain the cell attachment activity³, is enclosed in brackets. The one-letter code used to denote each amino acid is: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

single fibronectin molecule for the cell surface is so low that very high concentrations of soluble fibronectin or its fragments would be needed to overcome the cooperative interaction of many immobilized fibronectin molecules with cells. That this is the case has been suggested by the work of Grinnell and colleagues^{10,11} and by that of Hughes *et al.*¹².

The small peptides, in contrast to fibronectin and its larger fragments, are highly soluble, which makes it possible to test them at millimolar concentrations for inhibition of cell attachment. For these tests we chose peptides which did not have a cysteine residue to avoid possible binding of the peptide to the substrate by disulphide exchange. We found that the Gly-Arg-Gly-Asp-Ser-Pro and Arg-Gly-Asp-Ser peptides did indeed interfere with the attachment of NRK cells to fibronectin-coated substrates (Fig. 4a). (In experiments where they were compared directly, the Gly-Arg-Gly-Asp-Ser-Pro peptide promoted cell attachment to surfaces coated with it to the same extent as did Gly-Arg-Gly-Asp-Ser-Cys.) The inhibition by the peptides occurred, in a dose-dependent manner, at peptide concentrations $>1 \times 10^{-4}$ M, (Fig. 4b). An affinity constant for the cell surface of 3×10^{-4} M and 6×10^{-4} M for the hexapeptide and tetrapeptide, respectively, was obtained using the method for estimating antibody affinity described by Müller¹³. Peptides not containing the Arg-Gly-Asp-Ser sequence showed no inhibition of cell attachment to fibronectin, indicating that the inhibition is specific and that inclusion of peptides in the cell attachment assay at these concentrations had no adverse effects on the cells. Furthermore, the attachment of cells to concanavalin A (Sigma) remained unaffected by the Arg-Gly-Asp-Ser-containing peptides (data not shown).

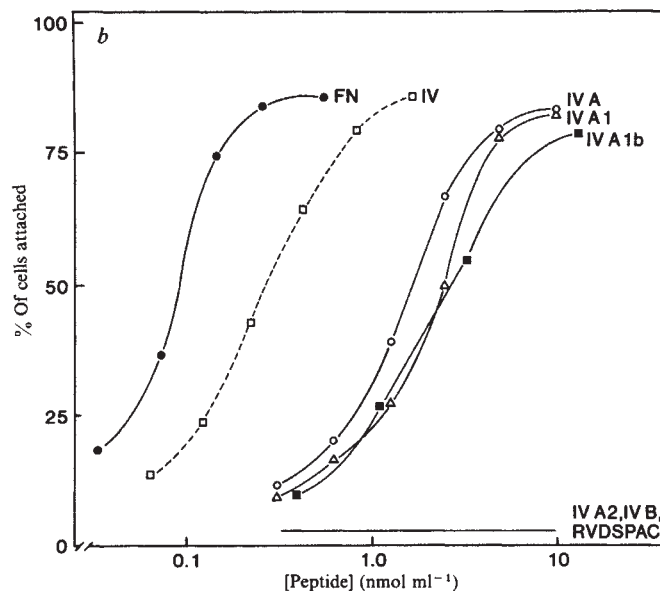
The tetrapeptide in other proteins

Having established the importance of the Arg-Gly-Asp-Ser sequence in fibronectin, we looked for this tetrapeptide in other proteins by searching the library of sequences compiled by the National Biomedical Research Foundation (George Washington University Medical Center, Washington, D.C.). This search revealed five proteins having the tetrapeptide (Table 1). Taking into account the frequency at which each of these four amino acids occurs in proteins and the 351,138 tetrapeptides examined from the sequence library, six such sequences would have been expected.

Of the five proteins having the active tetrapeptide sequence, fibrin(ogen) was of most immediate interest because of its demonstrated interaction with fibronectin¹⁴ and the platelet surface^{15,16}. Therefore, we tested a synthetic nonapeptide modelled on residues 568–580 of the α -chain of fibrinogen and found that it also promoted cell attachment Table 1 and Fig. 5). Attempts to demonstrate the attachment of our test cells to intact fibrinogen or fibrin, however, have not given clear-cut results. In accordance with earlier results¹⁷, no attachment of fibroblastic cells to fibrinogen insolubilized on plastic could be demonstrated. Attachment of cells to the fibrinogen or fibrin polypeptides, separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose as described elsewhere¹⁸, gave only weak or, at times, no attachment to the separated α -chain (data not shown). In contrast, the activity of fibronectin is readily detectable in both conditions. It may be that the cell attachment site in fibrinogen is cryptic or destroyed during the assay procedures, or that the surrounding sequences

Code	Sequence	Concentration for 50% cell attachment
Fibronectin		0.10 nmol ml ⁻¹
IV	Y A V T G R G D S P A S S K P I S I N Y R T E I D K P S Q M (C)	0.25
IVA	V T G R G D S P A S S K P I (C)	1.6
IVB	S I N Y R T E I D K P S Q M (C)	> 50.0
IVA1	V T G R G D S P A (C)	2.5
IVA2	S P A S S K P I S (C)	> 50.0
IVA1a	V T G R G D (C)	10.0
IVA1b	G R G D S (C)	3.0
IVA1c	R G D S P A (C)	6.0
RVDS	R V D S P A (C)	> 50.0

Fig. 2 Attachment of NRK cells to immobilized fibronectin and synthetic fibronectin peptides. **a**, Peptides were synthesized by Peninsula Laboratories (Belmont, California) according to our specifications, to include a COOH-terminal cysteine (C). The activity of each peptide is indicated on the right of the figure by the concentration necessary to achieve 50% maximal attachment in the assay described below. **b**, The synthetic peptides were assayed for their ability to promote the attachment of NRK cells by first attaching the peptides via the heterobifunctional cross-linker SPDP (*N*-succinimidyl-3-(2-pyridyldithio) propionate; Sigma) to rabbit IgG which had been immobilized on polystyrene³. By using radio-labelled peptides or antibodies to the larger peptides, we have determined that coupling of the peptides using this method is more than 85% complete (data not shown). Peptides not containing a cysteine are not included in the figure as the methods used to immobilize them appeared to be less efficient (see text). The attachment assay was carried out as described³⁵ using freshly trypsinized NRK cells. After incubation for 1 h at 37°C, those cells which had attached were fixed, stained and quantitated using either an Artek cell counter or a vertical pathway spectrophotometer. In all cases, maximum attachment was ~80–90% of the cells plated. The titration curves for whole fibronectin (FN) and peptide IV (ref. 3) are shown here for comparison.



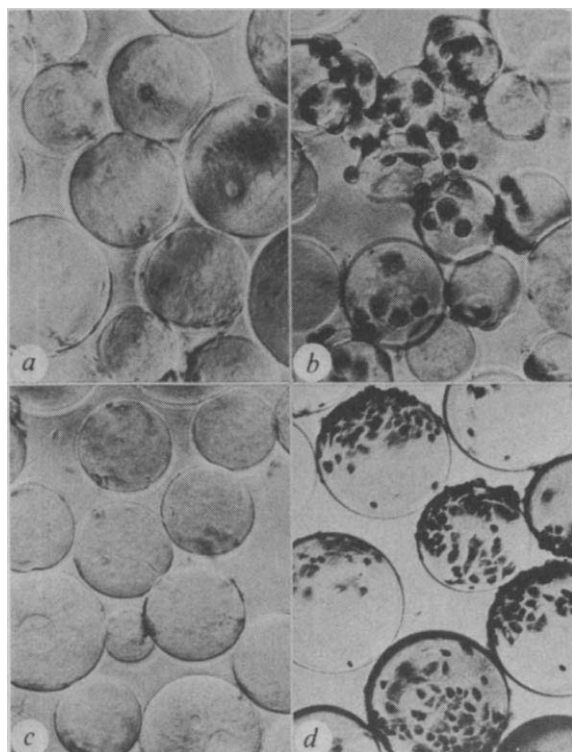


Fig. 3 Attachment of NRK cells to CH-Sepharose beads carrying tetrapeptides or fibronectin. Peptides TGRG (a), RGDS (b), GDSP (c) and fibronectin (d) were coupled to CH-Sepharose 4B (Pharmacia) by treatment of the Sepharose beads with 0.14 M 1-ethyl-3(3-dimethylaminopropyl)carbodiimide-HCl (Sigma) at pH 5–6 for 18 h at room temperature in the presence of a twofold molar excess of peptide relative to the available reactive groups on the beads. Coupling was quantitated by amino acid analysis and by monitoring the change in UV absorption. Each ml of Sepharose beads carried ~3–4 mg peptide or 10 mg of fibronectin. Attachment was assayed by incubating NRK cells with a monolayer of beads for 1 h at 37°C. The beads were then allowed to sediment (at 1 g) through 3 ml of 3% paraformaldehyde in phosphate-buffered saline to separate unattached cells and fix those cells which had attached. The unattached cells were decanted and the beads were stained with toluidine blue and observed under the microscope. a, b, and c $\times 50$; d $\times 40$.

in some way weaken the activity of this site so that it is not recognized by fibroblastic cells in the intact molecule. The Arg-Gly-Asp-Ser sequence in fibrinogen is flanked on both the COOH-terminal and NH₂-terminal sides by sequences which are more hydrophilic than the hydrophobic regions present on either side of this sequence in fibronectin. On the other hand, platelets interact with fibrinogen^{19,20} at more than one site within the molecule²¹, and they also interact with fibronectin²². Future work should show whether the Arg-Gly-Asp-Ser sequence of fibrin is recognized at any stage of haemostasis by platelets or other types of cells, or whether this sequence might become uncovered on enzymatic dissolution of a blood clot. Future work should also show whether this sequence mediates the interaction of any of the other proteins listed in Table 1.

Implications

Our data show that the minimum signal necessary for the binding of cells to fibronectin resides in the short amino acid sequence Arg-Gly-Asp-Ser and that each of the four amino acids contributes to the binding. That this sequence is common to a few other proteins which may interact with cells implies that cells may use a single mechanism (receptor?) for interacting with several proteins. Although no structural basis for the surprising activity of such a small peptide is readily apparent, some of its properties are suggestive. As already mentioned, the tetrapeptide is found in a segment of fibronectin that would be very likely to form a hydrophilic loop at the surface of the molecule^{8,9} and would thus be available to interact with cells. As in our hands polycations are relatively ineffective compared with the synthetic peptides in promoting the attachment of fibroblasts and even less effective in promoting cell spreading (M.D.P. and E.R., unpublished results), it is clear that binding is not due solely to the arginine residue. In any case, the other three residues of the tetrapeptide are clearly important because substitution of the glycine residue with valine eliminates cell attachment activity, and omission or substitution of the aspartate alters the activity of the peptide drastically (Fig. 4). Furthermore, we have tested a synthetic peptide taken from a sequence in collagen²³, Lys-Gly-Glu-Ser-Gly-Ser-Pro, and found no cell attachment promoting activity (data not shown), indicating that a mere conservation of the charge relationships (Lys-Gly-Glu... versus Arg-Gly-Asp...) does not maintain the activity.

We have also tested the activity of other peptides that resemble the Arg-Gly-Asp-Ser sequence and are present in collagens²³. One such sequence, Arg-Gly-Asp-Thr-Gly-Ala-Thr-Gly-Arg, (taken from type I collagen) promotes cell attachment almost as well as the fibronectin peptides; two other sequences (taken from collagens of different genetic types²³), Gln-Gly-Ile-Arg-Gly-Asp-Lys-Gly-Glu-Pro and Gly-Ser-Arg-Gly-Asp-Hyp-Gly-Thr-Hyp, when tested as synthetic peptides had no cell attachment-promoting activity at all (unpublished data). These results indicate that the serine residue is not essential but only conservative substitutions of this residue are compatible with activity. The sequences flanking the Arg-Gly-Asp-Ser sequence also affect activity as illustrated by the fact that the Gly-Arg-Gly-Asp-Ser-Pro peptide can inhibit cell attachment to fibronectin better than the Arg-Gly-Asp-Ser peptide.

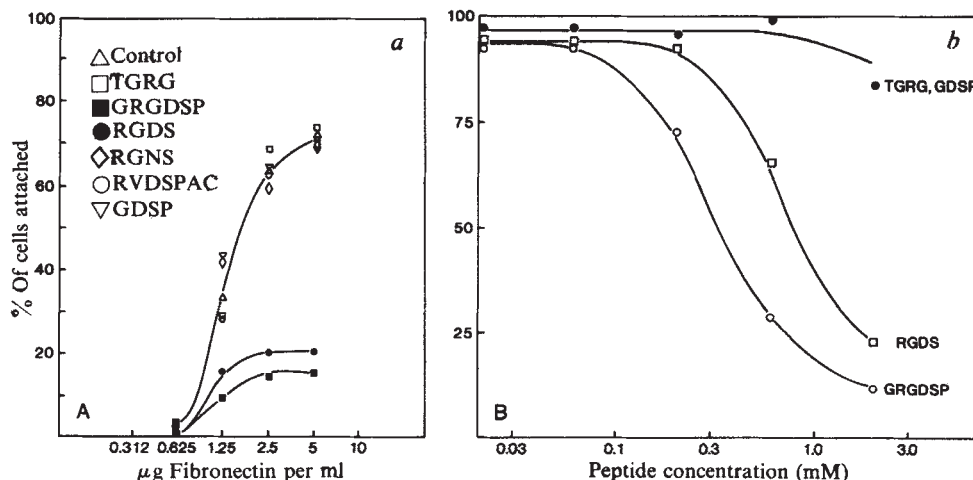
The data we have obtained by selective substitution within the recognition sequence reveal some interesting possibilities about modification of the fibronectin activity in cells and tissues. Based on these results, one can infer that a single point mutation in the fibronectin gene (for example, one that would change glycine to valine; Fig. 2) could destroy the activity of the recognition sequence. This raises the possibility that there exist fibronectins which are ineffective cell attachment promoters. Moreover, the cell attachment sequence may be subject to modification by phosphorylation. Fibronectin has been shown to carry covalently bound phosphate^{24–26}. A partially phosphorylated serine has been found elsewhere in the fibronectin molecule within a region not showing internal homology with the cell attachment domain²⁶. However, the phosphorylated serine is homologous

Table 1 Proteins having a sequence similar to the cell attachment-promoting sequence of fibronectin

Proteins	Sequence	Ref.	Cell attachment
Fibronectin	A V T G R G D S P A S S K	3	Active
Fibrinogen α -chain	T S Y N R G D S T F E S K	4	Active
λ Receptor on <i>E. coli</i>	G S F G R G D S D E W T F	5	NT
Sindbis coat protein	G V G G R G D S G R P I M	6	NT
α Lytic protease	A C M G R G D S G G S W I	33	NT
Testis-specific basic protein	K S R K R G D S A D R N Y	34	NT

A computer search through published protein sequences was conducted by The National Biomedical Research Foundation. The proteins having the sequence Arg-Gly-Asp-Ser are listed and the sequences flanking the tetrapeptide are shown. Residues identical to those found in the fibronectin sequence are boxed; chemically similar residues are enclosed in broken boxes; and sequences which were tested as synthetic peptides for cell attachment activity are shown in bold type. A synthetic nonapeptide was prepared from the fibrinogen sequence and was found to be active (see Fig. 5). The other proteins have not been tested (NT; see text for discussion of these proteins).

Fig. 4 Inhibition of attachment of NRK cells to immobilized fibronectin by synthetic fibronectin peptides. *a*, Microtitre wells were coated with decreasing concentrations of human plasma fibronectin, then non-bound fibronectin was washed away, and the attachment of NRK cells (10^4 cells per well) was assessed after incubation at 37°C for 45 min in the presence of each of the peptides shown, each at a concentration of 1 mM. *b*, The peptides also were titrated in wells coated with $5\ \mu\text{g ml}^{-1}$ fibronectin. NRK cells were again incubated at 37°C for 45 min, and attached cells were fixed, stained and counted. Incubation media for these experiments was Dulbecco's modified Eagle's medium (Flow) supplemented with glutamine-penicillin-streptomycin at 2 mM, 100 U and $100\ \mu\text{g ml}^{-1}$, respectively (Irvine Scientific) and bovine serum albumin $2\ \text{mg ml}^{-1}$ (Sigma).



to the serine in the cell attachment site (compare Arg-Glu-Asp-Ser- PO_3 with Arg-Gly-Asp-Ser). Extrapolating from the data presented here, one would expect that the Arg-Glu-Asp-Ser sequence would not show cell attachment activity (compare Arg-Glu-Asp-Ser with Arg-Val-Asp-Ser). This agrees with our finding only one cell attachment site in each fibronectin polypeptide¹. Whether the serine in the Arg-Gly-Asp-Ser sequence would be a suitable substrate for the same or a different protein kinase is unknown. In view of recent reports indicating the existence of cell surface protein kinase activity^{27,28} and the finding of increased phosphorylation of fibronectin from malignant transformed cells²⁹, this warrants further investigation. Phosphorylation could provide a means whereby cells might regulate their attachment and, where uncontrolled, could contribute to malignancy.

These results also raise the intriguing question of whether some prokaryotes and viruses use the fibronectin recognition sequence. The λ phage receptors on the surface of *E. coli* and Sindbis virus coat protein share a five-amino acid sequence with the cell attachment region of fibronectin (Table 1). It may be that some microorganisms use this sequence to interact with membranes of eukaryotic cells, and that some bacteriophages imitate the eukaryotic cell to gain entrance into *E. coli*.

The possibilities for future study discussed above are, at present, speculative. These possibilities, however, can be tested by using the peptides which inhibit cell attachment to fibronectin and, therefore, are also likely to interfere with other interactions which use this same recognition sequence. The peptides also provide us with the ability to specifically inhibit the cell attachment function of fibronectin, making it possible to study this function independent of the other functions of the protein in a variety of systems such as differentiation, cell motility, and in models of tumorigenesis and metastasis, all of which appear to be influenced by fibronectin³⁰⁻³².

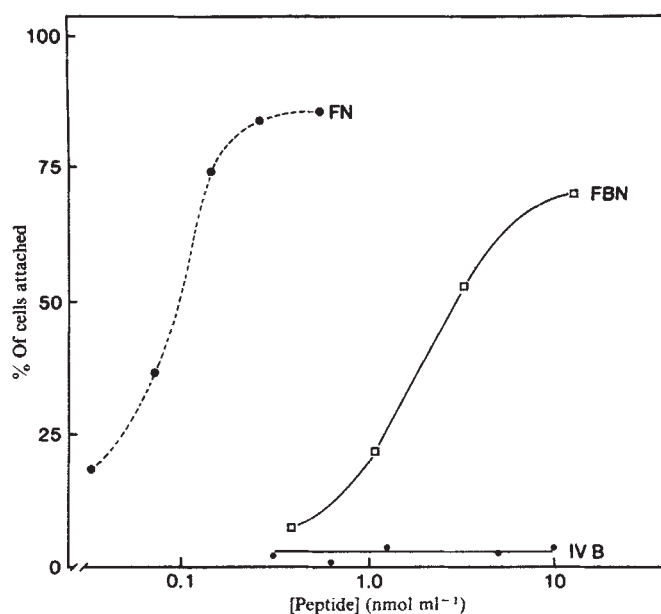


Fig. 5 Attachment of NRK cells to an immobilized synthetic peptide based on a sequence in fibrinogen. A synthetic fibrinogen peptide (FBN; see Table 1) was used to coat microtitre wells and was assayed for cell attachment as described in Fig. 2. Coating concentration of the peptide is indicated on the abscissa. The activities of fibronectin (FN) and peptide IVB are included for comparison.

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- Pierschbacher, M. D., Hayman, E. G. & Ruoslahti, E. *Cell* **26**, 259-267 (1981).
- Pierschbacher, M. D., Ruoslahti, E., Sundelin, J., Lind, P. & Peterson, P. A. *J. biol. Chem.* **257**, 9593-9597 (1982).
- Pierschbacher, M. D., Hayman, E. G. & Ruoslahti, E. *Proc. natn. Acad. Sci. U.S.A.* **80**, 1224-1227 (1983).
- Watt, K. W. K., Cottrell, B. A., Strong, D. D. & Doolittle, R. F. *Biochemistry* **18**, 5410-5416 (1979).
- Clement, J. M. & Hofnung, M. *Cell* **27**, 507-514 (1981).
- Rice, C. M. & Strauss, J. H. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2062-2066 (1981).
- Rose, G. D. *Nature* **272**, 568-590 (1978).
- Chou, P. & Fassman, G. D. *Adv. Enzym.* **47**, 145-147 (1978).
- Kyte, J. & Doolittle, R. F. *J. molec. Biol.* **157**, 105-132 (1982).
- Grinnell, F., Lang, B. R. & Phan, T. V. *Expl Cell Res.* **142**, 499-504 (1982).
- McAbee, D. D. & Grinnell, F. *J. Cell Biol.* **97**, 1515-1523 (1983).
- Hughes, R. C., Pena, S. D. J., Clark, J. & Dourmashkin, R. R. *Expl Cell Res.* **121**, 307-314 (1979).
- Müller, R. J. *J. immun. Meth.* **34**, 345-352 (1980).
- Ruoslahti, E. & Vaheri, A. *J. exp. Med.* **141**, 497-501 (1975).
- Plow, E. F. & Ginsberg, M. H. *J. biol. Chem.* **256**, 9477-9481 (1981).
- Plow, E. F. & Marguerie, G. *Proc. natn. Acad. Sci. U.S.A.* **79**, 3711-3715 (1982).
- Grinnell, F., Feld, M. & Minter, D. *Cell* **19**, 517-525 (1980).

- Hayman, E. G. *et al. J. Cell Biol.* **95**, 20-23 (1982).
- Marguerie, G. A., Plow, E. F. & Edgington, T. S. *J. biol. Chem.* **254**, 5357-5363 (1979).
- Bennett, J. S. & Vilare, G. *J. clin. Invest.* **64**, 1393-1401 (1979).
- Hawiger, J., Timmons, S., Kloczewiak, M., Strong, D. D. & Doolittle, R. F. *Proc. natn. Acad. Sci. U.S.A.* **79**, 2068-2071 (1980).
- Lahav, J., Schwarz, N. A. & Hynes, R. O. *Cell* **31**, 253-262 (1982).
- Miller, E. J. in *Connective Tissue Biochemistry* (eds Piez, K. A. & Reddi, A. H.) (Elsevier, Amsterdam, 1983).
- Teng, M.-H. & Rifkin, D. B. *J. Cell Biol.* **80**, 784-791 (1979).
- Ledger, P. W. & Tanzer, M. L. *J. biol. Chem.* **257**, 3890-3895 (1982).
- Petersen, T. E. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 137-141 (1983).
- Kubler, D., Pyerin, W. & Kinzel, V. *J. biol. Chem.* **257**, 322-329 (1982).
- Kubler, D., Pyerin, W., Burrow, E. & Kinzel, V. *Proc. natn. Acad. Sci. U.S.A.* **80**, 4021-4025 (1983).
- Ali, I. U. & Hunter, T. *J. biol. Chem.* **256**, 7671-7677 (1981).
- Hynes, R. O. & Yamada, K. M. *J. Cell Biol.* **95**, 369-377 (1982).
- Mosher, D. F. *Prog. Hemostasis Thromb.* **5**, 111-151 (1980).
- Ruoslahti, E., Engvall, E. & Hayman, E. G. *Coll. Res.* **1**, 95-128 (1981).
- Olson, M. O. J., Nagabhushan, N., Dzwiniel, M., Smillie, L. B. & Whitaker, D. R. *Nature* **228**, 438-442 (1970).
- Kistler, W. S., Noyes, C., Hsu, R. & Heinrichson, R. L. *J. biol. Chem.* **250**, 1847-1853 (1975).
- Ruoslahti, E., Hayman, E. G., Pierschbacher, M. & Engvall, E. *Meth. Enzym.* **82**, 803-831 (1982).

The Sunyaev–Zeldovich effect towards three clusters of galaxies

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An unequivocal detection of the Sunyaev–Zeldovich effect¹, the cooling of the brightness temperature of the microwave background radiation towards the atmospheres of clusters of galaxies, has profound cosmological significance^{2–6}, but has hitherto proved elusive. We present here measurements of the effect towards three rich clusters of galaxies made with the 40-m telescope of the Owens Valley Radio Observatory at 20.3 GHz. These results are three times more sensitive than other results previously reported^{7–9}, are free from systematic errors at the 100 μ K level, and provide very strong evidence for the reality of the Sunyaev–Zeldovich effect.

The consensus of observational data supports the presence of a Sunyaev–Zeldovich effect towards only three clusters of galaxies—0016+16, Abell 665 and Abell 2218—although the support is by no means unqualified^{7–9}. To provide further evidence for the reality or otherwise of the effect, we have worked for several years with the 40-m telescope of the Owens Valley Radio Observatory of the California Institute of Technology. Our results to 1983 January are reported elsewhere¹⁰, but here we present the results of a successful observing run at 20.3 GHz made during the period 21 December 1983–3 January 1984.

For these observations, the 40-m telescope was equipped with a tunable K-band maser receiver with bandwidth ~ 400 MHz which provided an overall system noise temperature ~ 40 K. At our observing frequency the 40-m telescope has a beam efficiency of about 0.48, and a half-power beamwidth of 107 arc s. Observations were made using a symmetrical beam-switching scheme¹⁰ with a beam-separation of 7.15 arc min. The weather was mostly good during our allocated observing period, and 237 h of useful data were obtained for the three clusters of galaxies 0016+16, Abell 665 and Abell 2218, and for nine reference regions of 'blank' sky located adjacent to the clusters or distant from them. These reference regions provide strong controls on the presence of systematic errors, which have severely handicapped earlier observations¹¹. The data obtained from these observations were reduced using a modified form of the analysis of the variance¹²: tests with partial data-sets analysed in different ways gave consistent results. A test for parallactic-angle dependent systematic errors was also made¹⁰, but none was found.

The results of these observations are given in Table 1, which contains the results for all the clusters and reference regions observed. The N and S positions associated with the clusters refer to points 7 arc min north and south of the cluster centres (the C positions, whose coordinates are given in ref. 10) respectively. The 0016+16 S position is contaminated by the presence of a nearby radio source whose contribution to the brightness temperature at this location is calculated (from lower-frequency measurements) to be 510 ± 120 μ K (refs 9, 13 and R. Laing, personal communication), and the brightness temperature measurement at this point has been corrected for this contribution. No corrections for the presence of radio sources have been applied to any of the other results in Table 1.

Because these observations were made using symmetrical beam-switching in azimuth, the ΔT_{RJ} brightness temperature measurements given in Table 1 represent the differences between

the brightness temperatures towards the nominal points observed and the averages of points located on 'reference arcs' one beam-separation away, convolved with the primary beam-pattern of the telescope. For each cluster centre, the ΔT_{RJ} value therefore represents a measurement of a constant signal of ~ 0.45 of the central microwave decrement, ΔT_0 (using X-ray core sizes, θ_{cx} , of 0.50, 0.92 and 0.97 arc min for 0016+16, Abell 665 and Abell 2218 respectively, and a model

$$\Delta T_{RJ}(\theta) = \Delta T_0(1 + \theta^2/\theta_{cx}^2)^{-1/4}$$

for the angular distribution of the decrement¹⁰). For the N and S reference locations, the reference arcs are located asymmetrically with respect to the cluster centres, and hence the cluster gas distributions, and cross the cluster centres at parallactic angle 90°. Averaged over the observational parallactic angle coverage, this implies that the N and S reference positions in these three clusters should see signals of only a few per cent of ΔT_0 appearing as small decrements or enhancements in the brightness temperature of the background radiation.

The differences between the values of ΔT_{RJ} measured at the cluster centres and at the N and S reference positions should, therefore, represent between 0.42 and 0.48 ΔT_0 , and the significant depression of the C-position values of ΔT_{RJ} below those measured N and S of the cluster centres demonstrates the existence of significant Sunyaev–Zeldovich coolings towards each of the three clusters of galaxies.

Taking account of the parallactic-angle coverages of the observations, we can estimate for each cluster both the central microwave background decrement, ΔT_0 , and any residual (constant) systematic offset signal, ΔT_s . These are given in Table 2. The values of ΔT_s are consistent with our limit of 100 μ K deduced from the blank-sky results, but differ from zero at the 10% significance level even when correlations between ΔT_s and ΔT_0 in the fit are taken into account. For comparison Table 2 also gives the values of ΔT_0 deduced on the assumption that $\Delta T_s = 0$. Some small ΔT_s might be caused by pointing uncertainties or by the use of an inadequate model for the cluster atmospheres. Independent evidence for ~ 100 μ K offsets has been found in other sensitive observations of the microwave background radiation using the 40-m telescope (A. C. S. Readhead, personal communication).

The values of ΔT_0 given in Table 2 correspond to significant detections of the Sunyaev–Zeldovich effect in each of these three clusters. The consequences of the detection of decrements with $\Delta T_0 = -0.7$ to -1.4 mK have been discussed elsewhere¹⁰—we note in particular that the very significant (8σ) detection of a

Table 1 The results

Cluster	Position	Brightness temperature change, ΔT_{RJ} (μ K)	χ^2	Degrees of freedom	Fraction of data rejected in analysis
0016+16	C	-644 ± 79	53.8	44	0.00
	N	$+103 \pm 93$	11.8	8	0.11
	S	$-100 \pm 146^*$	17.0	10	0.15
Abell 665	C	-335 ± 48	46.6	45	0.00
	N	-55 ± 63	17.9	13	0.00
Abell 2218	S	-131 ± 57	12.9	11	0.02
	C	-338 ± 48	73.0	57	0.00
	N	$+9 \pm 58$	23.0	15	0.00
Blank sky	S	-115 ± 69	14.2	10	0.00
	1	$+6 \pm 74$	48.1	23	0.00
	3	$+189 \pm 86$	39.1	26	0.00
	5	-126 ± 96	21.4	9	0.00

The coordinates of the cluster centres, and of the blank sky regions, are given in ref. 10.

* $+410 \pm 84$ μ K before correction for the radio source.

Table 2 Central microwave background decrements and systematic offsets

Cluster	Systematic offset signal ($\Delta T_s/\mu\text{K}$)	Central microwave background decrement with $\Delta T_s \neq 0$ ($\Delta T_0/\mu\text{K}$)	Central microwave background decrement with $\Delta T_s = 0$ ($\Delta T_0/\mu\text{K}$)
0016 + 16	+79 $\pm$ 82	-1,584 $\pm$ 256	-1,402 $\pm$ 172
Abell 665	-89 $\pm$ 44	-503 $\pm$ 136	-694 $\pm$ 98
Abell 2218	-40 $\pm$ 47	-507 $\pm$ 143	-700 $\pm$ 98

microwave background decrement towards 0016 + 16 may provide a useful cosmological test²⁻⁶.

We conclude that there should be no further doubt of the reality of the Sunyaev-Zeldovich effect in these three clusters of galaxies.

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Received 17 February; accepted 12 March 1984.

1. Sunyaev, R. A. & Zeldovich, Ya. B. *Compts. Astrophys. Space Phys.* **4**, 173-178 (1972).
2. Gunn, J. E. *Observational Cosmology* (eds Maeder, L. & Tammann, G.) 62 (Geneva Observatory, 1978).
3. White, S. D. M. & Silk, J. I. *Astrophys. J. Lett.* **226**, L103-106 (1978).
4. Birkinshaw, M. *Mon. Not. R. astr. Soc.* **187**, 847-862 (1979).
5. Fabbri, R., Melchiorri, F., Mencaraglia, F. & Natale, V. *Astr. Astrophys.* **74**, L20-24 (1979).
6. Cavaliere, A., Danese, L. & De Zotti, G. *Astr. Astrophys.* **75**, 322-325 (1979).
7. Birkinshaw, M. *Clustering in the Universe* (eds Gerbal, D. & Mazure, A.) 265 (Editions Frontières, Paris, 1983).
8. Meyer, S. A., Jeffries, A. D. & Weiss, R. *Astrophys. J. Lett.* **271**, L1-4 (1983).
9. Andernach, H., Schallwisch, D., Sholomitski, S. & Wielebinski, R. *Astr. Astrophys.* **124**, 326-330 (1983).
10. Birkinshaw, M. & Gull, S. F. *Mon. Not. R. astr. Soc.* **206**, 359-375 (1984).
11. Lake, G. & Partridge, R. B. *Astrophys. J.* **237**, 378-389 (1980).
12. Birkinshaw, M., Gull, S. F. & Northover, K. J. E. *Mon. Not. R. astr. Soc.* **197**, 571-592 (1981).
13. Birkinshaw, M., Gull, S. F. & Moffet, A. T. *Astrophys. J. Lett.* **251**, L69-73 (1981).

Alignment of distant radio sources

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A typical extragalactic radio source is a simple double or elongated structure with a well-defined orientation on the sky. In 1972, Willson¹ investigated the relative orientation of 74 extragalactic 3C radio sources mapped at the Cambridge 1-mile telescope, and noted that extended extragalactic radio sources tend to be parallel if they are separated by less than 10° on the sky. Since 1972, many more radio sources have been mapped with resolutions 10-20 times greater than that of the observations available to Willson. I have therefore reinvestigated this problem in two separate and largely independent samples of some 300 sources each, and report here that the alignment is present to a high significance level, provided that only the more distant sources are considered. The effect could be due to gravitational imaging if most of the matter in a closed universe is in thin filaments.

In brief, the technique used by Willson is as follows. For a pair of radio sources one first determines the angular distance, D , measured along the great circle connecting the sources. If the axes of the radio sources make angles α and β with the connecting great circle, then the angle between the radio sources is defined as $X = \alpha - \beta$ reduced to the interval $0, \pi/2$. If $X = 0$,

the sources are parallel; if $X = \pi/2$ they are perpendicular. Given N sources with measured position angles, one may calculate $\bar{X}(D_1, D_2)$ which is the mean value of X for all n_p pairs with separations between D_1 and D_2 . If there exists no correlation of position angles, the distribution of X is uniform between 0 and $\pi/2$, and $\bar{X}(D_1, D_2) = 45^\circ \pm 45^\circ/\sqrt{3n_p}$.

Willson found that for all pairs with $D > 10^\circ$ in his sample, $42^\circ < \bar{X}(D) < 50^\circ$. But for close pairs, $\bar{X}(D < 10^\circ) \approx 36^\circ$, which, given his relatively small number of close pairs was about two standard deviations below the expected mean of 45° . The probability of a 2σ fluctuation in his 11 separation intervals is about 10% which is, roughly, the significance of the effect.

The first of the samples studied here consists of published maps of identified extragalactic radio sources. The sample contains most of the 3C objects listed in the compilation of Laing *et al.*² with the position angles taken from the references therein, primarily from the Cambridge 5-km surveys³⁻⁷. The sample also contains objects from a published quasar survey⁸ (mostly 4C objects) including more than 100 quasars observed at the Very Large Array (VLA) at resolutions of 1-2 arc s as well as a number of additional quasars compiled from earlier surveys⁹⁻¹¹. Source position angles were measured directly from the maps in most cases, and strongly distorted sources with ambiguous position angles were excluded. Following Willson an additional selection criterion is applied to objects mapped with one-dimensional arrays. A source is removed from the sample if its angular size is smaller than two north-south half-power beamwidths because inclusion of such sources would introduce an east-west bias in the position angle distribution.

With these conditions the total sample consists of 320 sources, of which 177 are identified with quasars and 143 with radio galaxies. Carrying out the analysis described above, I find only a very marginal effect. For separations smaller than 5° , $\bar{X}(D < 5^\circ) = 42.76^\circ$, which is a fluctuation of 1.33σ below the expected value of 45° . But considering only the sources with redshifts greater than 0.4 (191 sources) the results are quite different, as seen in Fig. 1 which is $\bar{X}(D)$ binned in 5° intervals. Here we find that $\bar{X}(D < 5^\circ) = 36.37^\circ$, which is a deviation of 3.4σ . The distribution of X for all sources with separations smaller than 5° is shown in Fig. 2. This distribution obviously deviates significantly from uniform with more sources tending to be parallel. From the Kolmogorov-Smirnov test¹² the probability that the

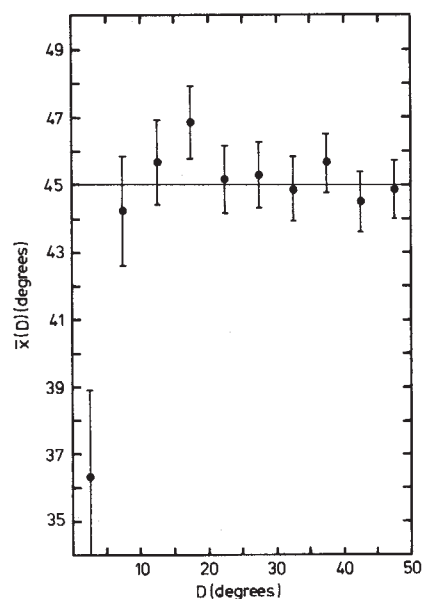


Fig. 1 The mean angle between radio sources in degrees as a function of angular separation in degrees for all sources in the 3C-4C sample with $z > 0.4$. The average is taken over 5° intervals and the error bars indicate the $\pm$ standard deviation for a uniform distribution ($45^\circ/\sqrt{3n_p}$ where n_p is the number of pairs in a bin).

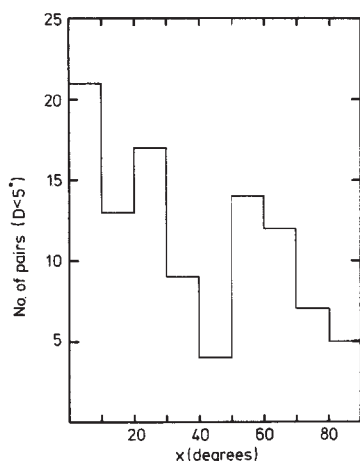


Fig. 2 The distribution of X , the angle between sources, for 102 pairs of sources in the 3C-4C sample closer than 5° on the sky and with $z > 0.4$.

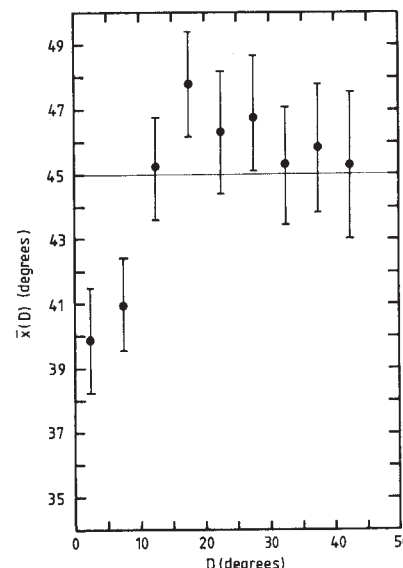


Fig. 3 The same as Fig. 1, but for all sources in the B3 sample smaller than 42 arc s.

observed distribution of angles between closely separated sources is drawn from a uniform random distribution is 0.003. This has been verified by Monte Carlo simulations in which source positions are unchanged but position angles are scrambled. The probability of finding such a large deviation over the entire range of separations (0° – 50°) is roughly 10 times larger or about 3%.

As all sources in this 3C-4C sample have measured or estimated redshifts, it is possible to determine whether or not sources in nearly parallel pairs at close separations are at the same distance. There are 34 pairs in which the sources are closer than 5° and are parallel to within 20° (that is, $D < 5^\circ$, $X < 20^\circ$). The distribution of Δz , the redshift difference between the two members of these pairs, does not differ significantly from that of 34 pairs drawn randomly from the sample. For only four of these pairs is $\Delta z < 0.1$. This implies that the nearly parallel sources with $D < 5^\circ$ which produce the effect are in general not physically close together.

A second largely independent and more homogeneous sample is provided by recent VLA observations of more than 1,000 sources selected from the third Bologna survey (B3) (R. Perley, B. G. Clark, A. H. Bridle and G. Greuff, in preparation). All mapped sources lie within a narrow declination range (38° – 48°), and the observations were done in a mode which assured a circular beam with a half-power width of 14 arc s. Therefore there should be no systematic false effects resulting from an elongated beam. Of the mapped sources roughly one-third have structure on a scale larger than the beam. Position angles were measured for 297 sources with simple double or linear structure. Most of the sources in this sample are unidentified so redshift restrictions cannot be made.

In the complete sample there is no tendency for alignment. For separations less than 5° , we find $\bar{X}(D < 5^\circ) = 44.66^\circ$, which is within 1 s.d. of the expected value. Although a redshift restriction is not possible for the B3 sample, an angular size restriction is in some sense equivalent. By eliminating large sources from the sample, one eliminates nearer sources although the sample is still contaminated by small nearby sources. If all sources larger than 42 arc s (three beam widths) are removed, the results are shown in Fig. 3 which again is $\bar{X}(D)$ averaged in 5° bins. Although there are only 94 sources smaller than 42 arc s, there are still many close pairs because of the very restricted sky coverage of the survey. Here again we see a very significant tendency toward parallelism at small separations: $\bar{X}(D < 5^\circ) = 39.84^\circ$, or a 3.2σ deviation. Moreover, in this sample, the effect of alignment seems to extend to 10° with $\bar{X}(D < 10^\circ) = 40.48^\circ$, a 4.3σ deviation. The distribution of X for pairs separated by less than 10° deviates from uniform at the level of 7×10^{-5} (data not shown). The probability for finding such a large deviation at any separation is 0.1%.

Any subtle instrumental effect which might produce a spurious alignment (such as, for example, some aspect of the telescope side-lobe pattern giving greater sensitivity for extended structure in certain orientations) would be expected to show up as a non-randomness in the distribution of position angles. In both restricted samples taken separately the distributions of position angles do deviate somewhat from uniform at significance levels of about 10%. To test whether or not non-uniformity of the position angle distributions of this order could result in apparent alignment of closely separated sources, I have generated random skies in which the overall distribution of position angles is exactly the same as that observed; that is, the position angles are not scrambled but redistributed among sources. The frequency of chance alignments as significant as those observed is less than $1/1,000$ in both samples implying that the effect does not result from non-uniformity in the global position angle distribution. It is more likely that the small non-randomness seen in the position angle distribution is an effect of the presence of several groups of closely separated well-aligned sources in samples of 100 to 200 objects.

The fact that the very pairs of objects which give the effect its high significance are widely separated in space (at least in the 3C-4C sample) implies that the effect arises along the line-of-sight. A possible explanation is gravitational imaging by superclusters¹³. Superclusters seem to be generally linear structures 10 to 100 Mpc in length¹⁴. The amplification produced by linear lenses is one-dimensional; the image of a background source is stretched in a direction perpendicular to the supercluster axis. One-dimensional amplification of a group of randomly oriented background sources can produce significant net alignment if most of the matter in an $\Omega_0 = 1$ universe lies in thin (< 1 Mpc) long (~ 50 Mpc) filaments¹⁵.

In view of the possible cosmological implications it is imperative that the effect should be confirmed. Remapping of the B3 radio sources at a resolution of 1–2 arc s is underway at the VLA and should definitely establish (or rule out) the reality of the apparent alignment. An additional experiment would consist of mapping all the radio sources down to some low flux limit in a small region of the sky (a $3^\circ \times 3^\circ$ field for example). If the effect is real, then the distribution of source position angles should deviate significantly from uniform.

Much of this work was done while I was visiting the NRAO in Charlottesville, Virginia. I thank M. S. Roberts for his hospitality and R. Perley and B. G. Clark at the VLA for providing their unpublished maps of B3 sources. I also thank A. H. Bridle, R. D. Ekers, J. H. Oort, T. Oosterloo, and especially T. S. van Albada for useful discussions and advice.

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- Willson, M. A. G. *Mon. Not. R. astr. Soc.* **155**, 275–282 (1972).
- Laing, R. A., Riley, J. M. & Longair, M. S. *Mon. Not. R. astr. Soc.* **204**, 151–187 (1983).
- Pooley, G. G. & Henbest, S. N. *Mon. Not. R. astr. Soc.* **169**, 477–526 (1974).
- Riley, J. M. & Pooley, G. G. *Mem. R. astr. Soc.* **80**, 105–137 (1975).
- Jenkins, C. J., Pooley, G. G. & Riley, J. M. *Mem. R. astr. Soc.* **84**, 61–99 (1977).
- Laing, R. A. *Mon. Not. R. astr. Soc.* **194**, 301–330 (1981).
- Laing, R. A. *Mon. Not. R. astr. Soc.* **195**, 261–324 (1981).
- Hintzen, P., Ulvestad, J. & Owen, F. *Astr. J.* **88**, 709–758 (1983).
- Miley, G. & Hartsuiker, A. *Astr. Astrophys. Suppl.* **34**, 129–163 (1983).
- Owen, F., Porcas, R. & Neff, S. *Astr. J.* **83**, 1009–1020 (1979).
- Potash, R. & Wardle, J. *Astr. J.* **84**, 707–717 (1979).
- Siegel, S. *Non-parametric Statistics* (McGraw-Hill, New York, 1956).
- Sanders, R. H., van Albada, T. S. & Oosterloo, T. *Astrophys. J.* (in the press).
- Oort, J. H. A. *Rev. Astr. Astrophys.* **21**, 373–428 (1983).

The case for antiparticles in the extragalactic cosmic radiation

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The presence of an excess of low-energy antiprotons in the primary cosmic radiation^{1–3} has given rise to several possible explanations⁴, some of which involve exotic processes such as mini-black holes and extragalactic antiparticles. Here we consider the latter possibility⁵ and show that there are interesting implications for the cosmic radiation at higher energies. Indeed, it may be possible to account for a previously puzzling feature of the cosmic ray spectrum—a ‘bump’ in the range 10^{14} – 10^{15} eV—by hypothesizing a primary extragalactic origin for the bulk of the observed cosmic ray antiprotons, although such an explanation is not unique. In this model most of the cosmic rays above 10^{15} eV are extragalactic. We prescribe a method of testing this hypothesis experimentally.

One of the most fundamental questions in cosmology is the question of the existence of antimatter in significant quantities in the Universe. Does antimatter have an equal role with matter in the makeup of the galaxies? This question has now become one of fundamental importance to physics also. In the contemporary paradigm of grand unified gauge theories it is related to the question of the nature of charge parity violation at high energies⁶. Early attempts at constructing a baryon symmetric cosmology^{7,8} suffered from the difficulty of providing an effective mechanism for separating matter and antimatter on ultimately large enough scales to be consistent with astronomical observations. However, recent theoretical work based on the concepts of grand unified theories (GUTs) has led to the development of a plausible baryon–antibaryon domain theory in which matter and antimatter are created in separate regions of survivable size to begin with (refs 9–11 and A. Mohanty, in preparation). Various observational aspects of this theory have been discussed previously^{12,13} and the subject of baryon symmetric cosmology has been reviewed recently^{14,15}. It should, of course, be noted that regardless of the ultimate fate of our present theoretical ideas either in favour of or against the reality of baryon symmetry on an overall universal scale, the experimental search for primary cosmologically significant quantities of antimatter is a task of deep significance. Further, both the recent theoretical work and the reports by various groups of surprisingly large fluxes of antiprotons in the cosmic radiation give immediacy to this search.

The present status of cosmic ray antiproton ($\bar{p}$) measurements and the attempts to understand them have been recently reviewed⁴ and an exegesis of the primary extragalactic origin

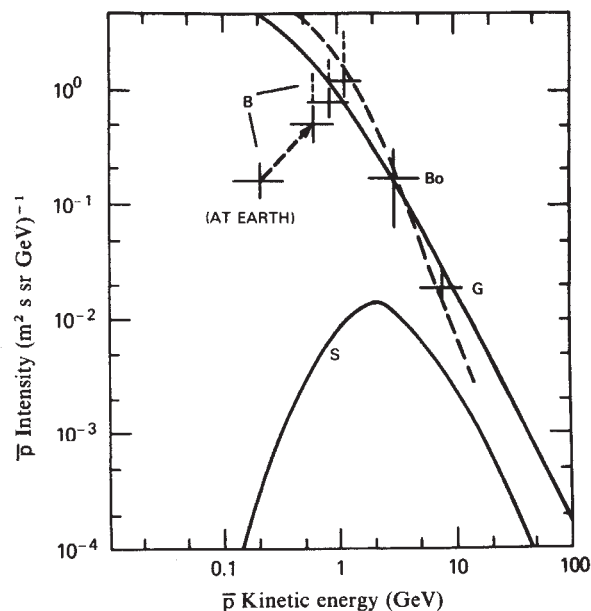


Fig. 1 Observations and theoretical $\bar{p}$ spectra. The data points are: B, from Buffington *et al.*³; Bo, from Bogomolov *et al.*²; and G, from Golden *et al.*¹. The higher points marked B take account of solar modulation effects²⁴. The solid curve (S) is for the secondary $\bar{p}$ production model with mean propagation length 5 g cm^{-2} , and the dashed line corresponds to the shape of the $\bar{p}$ spectrum calculated for a primordial black hole model²⁴. The higher solid line corresponds to our spectrum for the extragalactic primary hypothesis as discussed in the text.

hypothesis has also been given recently⁵. Here we will discuss further implications of potential basic importance to cosmic ray research and we will propose an experimental search programme based on these considerations. We start with the hypothesis that the baryon symmetric domain cosmology leads to a flux of extragalactic cosmic rays consisting of roughly equal amounts of protons and antiprotons with the sources of these cosmic rays being primarily active galaxies^{16,17} and with helium and antihelium nuclei being suppressed by destruction processes in these sources⁵. (We will also assume that helium and antihelium nuclei exist in the extragalactic cosmic rays at a lower level, supplied by leakage from normal matter galaxies and normal antimatter galaxies.) We further assume that the galactic wind is too weak to keep out the extragalactic cosmic radiation. Observations favour the interpretation that the galactic wind is in reality a ‘breeze’¹⁸.

Let us first consider the energy spectrum of the cosmic radiation. The measured spectrum of cosmic radiation can be represented by a power law in energy of the form $KE^{-\Gamma}$ with the spectral index $\Gamma \approx 2.75$ for several decades above the 10 GeV energy level. It seems likely that this radiation is produced primarily in galactic sources^{19,20}. Furthermore, the source spectrum of this radiation is expected to have a lower spectral index, Γ_s , than that observed at the Earth which has been steepened by energy-dependent propagation effects. A value for Γ_s of ~ 2.0 to 2.2 appears to be likely for two reasons. (1) Measurements of the ratio of secondary to primary nuclei in the cosmic radiation suggest that the mean lifetime in the Galaxy due to trapping by the tangled galactic magnetic fields falls with energy as $E^{-\delta}$ where the most recently derived value²¹ of δ is about 0.7. (2) The theoretical shock acceleration models for cosmic ray production that are presently favoured²² generally yield production spectra with Γ_s close to 2.

If we assume that there exists a general acceleration mechanism for generating cosmic rays which acts in both galactic and extragalactic sources to give a universal source spectrum with $\Gamma_s \approx 2$, as is now thought to be the case with shock acceleration, the extragalactic cosmic ray component should reflect this source

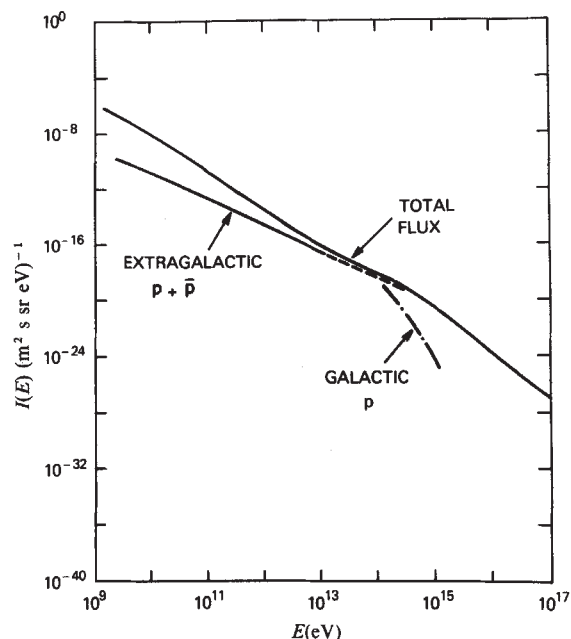


Fig. 2 The effect of extragalactic primary protons and antiprotons on the total cosmic ray spectrum according to the model discussed in the text. It can be seen that this model may account for the putative flattening in the observed cosmic ray spectrum near 10^{14} eV. $I(E)$ is the particle intensity, in the units shown.

spectrum. Thus, with the antiprotons assumed to be both primary and extragalactic and the bulk of the protons assumed to be galactic, the expected ratio of antiprotons to protons should increase with energy as E^δ . Taking $\delta = 0.7$, antiprotons could make up approximately 1% of the cosmic ray flux at an energy of ≈ 500 GeV and even $\sim 50\%$ at higher energies; this has important observational implications which we will discuss later. Figure 1 shows the present data on antiproton intensities and a theoretical extrapolation corresponding to a primary extragalactic antiproton flux with $\Gamma = 2.0$. For these considerations, we assume that secondary production of antiprotons is unimportant (see, for example, the estimate given in Fig. 1).

Figure 2 shows the effect of extrapolating the extragalactic intensity of both protons and antiprotons (this introduces a factor of two) with a spectral index of 2 to higher energies and superposing it on the galactic cosmic ray spectrum with index $\Gamma \approx 2.75$. Note that such an extrapolation implies that the extragalactic and galactic cosmic ray fluxes become comparable at an energy of $\sim 10^5$ GeV and that extragalactic particles predominate above this level. It is interesting to note that the resultant flattening in the spectrum occurs at this particular energy (10^5 GeV) where there have been claims²³ of a flattening in the cosmic ray spectrum as inferred from measurements of extensive air showers. It should, of course, be noted that a steepening in the spectra of both the galactic and extragalactic components would be required by the observations for energies above 10^6 GeV but this is not difficult to explain.

As our model indicates that the antiproton-to-proton ratio should increase with energy, measurements of the sign of the charges of cosmic rays at the highest practical energy and the determination of the spectra of the various charged components of the cosmic radiation up to that energy will provide a test of our hypothesis. Such a test requires that the experiment be placed above the atmosphere so that the incoming cosmic ray nuclei can be measured directly. Furthermore, the sign of the charged particles (and their magnitude) may be measured by using a superconducting magnet. Such an experiment, with an attainable energy of ~ 500 – $1,000$ GeV, could be flown aboard a space shuttle (T. Bowen *et al.*, unpublished NASA research proposal). In addition, an emulsion stack experiment could be flown on a high-altitude balloon or on the space shuttle to look

for antihelium nuclei, even at the reduced level implied by our hypothesis. A polar or near-polar orbit would be desirable to avoid the geomagnetic cutoff. In view of the almost impossible odds of creating a secondary ^4He antinucleus, the unambiguous detection of even one such particle would provide irrefutable evidence of primary cosmic ray antimatter. (It should be noted also that the production of 100–300 MeV antiprotons is extremely difficult because of well known kinematical factors⁵. This feat has proved elusive even to accelerator physicists who have deliberately set out in the laboratory to make such particles (C. Rubbia, personal communication). Thus, the observed low-energy antiprotons in the cosmic radiation are also quite difficult to explain as secondaries from cosmic-ray interactions.)

The experimental programme proposed above should add to our basic understanding of $\bar{p}$ origin. If the $\bar{p}/p$ ratio is observed to increase as $\sim E^{0.7}$, our hypothesis of extragalactic antiprotons from antimatter galaxies will receive strong support. The observation of antihelium nuclei would be conclusive. The extent to which non-observation of antihelium disproves our hypothesis is unclear, but if $\bar{\alpha}/\alpha < 10^{-5}$ (the value expected very approximately on the basis of $\bar{\alpha}$ s leaking from 'normal' antimatter galaxies), the difficulty would be severe.

Non-observation of antihelium (at the level given above), together with an increase in the $\bar{p}/p$ ratio with energy at a slower rate than E^δ (for example, $\propto E^{0.25}$ between 10 and 100 GeV), would be consistent with the closed galaxy model (or source trapping), although there are severe theoretical problems^{5,24} with such a model.

Finally, the observation of a $\bar{p}/p$ ratio falling rapidly with increasing energy would support the black hole model of Kiraly *et al.*²⁴ (see Fig. 1).

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1. Golden, R. L. *et al. Phys. Rev. Lett.* **43**, 1196–1199 (1979).
2. Bogomolov, E. A. *et al. Proc. 16th int. Cosmic Ray Conf.* **1**, 330–335 (1979).
3. Buffington, A. *et al. Astrophys. J.* **248**, 1179–1193 (1981).
4. Protheroe, R. J. in *Composition and Origin of Cosmic Rays* (ed. Shapiro, M. M.) 119–124 (Reidel, Dordrecht, 1983).
5. Stecker, F. W., Protheroe, R. J. & Kazanas, D. *Astrophys. Space Sci.* **96**, 171–177 (1983).
6. Senjanović, G. & Stecker, F. W. *Phys. Lett.* **96B**, 285–288 (1980).
7. Alfven, H. *Rev. mod. Phys.* **37**, 652–665 (1965).
8. Omnes, R. *Phys. Rep.* **3**, 1–56 (1972).
9. Brown, R. W. & Stecker, F. W. *Phys. Rev. Lett.* **43**, 315–318 (1979).
10. Sato, K. *Phys. Lett.* **99B**, 66–70 (1981).
11. Kuzmin, V. A., Tkachev, I. I. & Shaposhnikov, M. E. *Phys. Lett.* **105B**, 167–170 (1981).
12. Stecker, F. W. *Ann. N.Y. Acad. Sci.* **375**, 69–89 (1981).
13. Stecker, F. W. in *Progress in Cosmology* (ed. Wolfendale, A. W.) (Reidel, Dordrecht, 1982).
14. Chechetkin, V. M., Khlopov, M. Yu. & Sapozhnikov, M. G. *Rev. Nuovo Cimento* **5**, 1–100 (1982).
15. Stecker, F. W. in *Early Evolution of the Universe and Its Present Structure* (eds Abell, G. O. & Chincarini, G.) 1–79 (Reidel, Dordrecht, 1983).
16. Ginzburg, V. L. & Syrovatskii, S. I. *The Origin of Cosmic Rays* (Pergamon, London, 1984).
17. Hayakawa, S. *Cosmic Ray Physics* (Wiley, New York, 1969).
18. Jones, F. C. *Astrophys. J.* **229**, 747–752 (1979).
19. Dodds, D., Strong, A. W. & Wolfendale, A. W. *Mon. Not. R. astr. Soc.* **171**, 569–577 (1975).
20. Stecker, F. W. *Phys. Rev. Lett.* **35**, 188–191 (1975).
21. Ormes, J. F. & Protheroe, R. J. *Astrophys. J.* **272**, 756–764 (1983).
22. Drury, I., Axford, W. I. & Summers, D. *Mon. Not. R. astr. Soc.* **198**, 833–841 (1982).
23. Kempa, J., Wdowczyk, J. & Wolfendale, A. W. *J. Phys. A*, **7**, 1213–1221 (1974).
24. Kiraly, P. *et al. Nature* **293**, 120–122 (1981).

Neutrino tomography: Tevatron mapping versus the neutrino sky

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Volkova and Zatsepin¹ were the first to propose the use of a 'Tevatron' (a high-energy accelerator at 10^{12} eV or TeV energies) to beam neutrinos ν through the Earth to a mobile detector and obtain X rays of the planet's interior. The basic idea has been contingent for years on the opacity of the Earth to neutrinos at appropriate energies (10 – 10^3 TeV). This depends significantly on the neutrino–nucleon scattering cross-section, which in turn depends on the existence of an intermedi-

ate vector boson²⁻⁴, the acceptance of what physicists refer to as the standard model⁵⁻⁷ in high-energy physics, and finally the mass M_W of the intermediate W boson in that model. The announcement by CERN of the W boson mass^{8,9} ($M_W \sim 80$ GeV) permits us now to draw certain conclusions. Although we shall demonstrate that neutrino tomography at planetary densities is feasible, tomographic scanning schemes appear unrealistic in several respects. We shall address two sources of neutrinos for tomographic mapping of the densities, the Earth-based Tevatron and the neutrino sky.

Based on arguments from computerized tomography¹⁰⁻¹³, group discussions¹⁴ of future elementary particle facilities¹⁵ (also in presentations of satellite tomography, Fig. 1) pointed out that 'X-raying' the Earth with neutrinos cannot provide a proper solution to its composition and density for the same reasons that single-projection X rays do not constitute an adequate reconstruction for noninvasive imaging in medicine. Geophysical densities follow from the mapping of the Radon or Fourier transform of certain neutrino projections, and not from the Volkova-Zatsepin scheme, as we shall demonstrate.

Tomographic mapping of the Earth is illustrated in Fig. 1, using deep underwater muon and neutrino detectors¹⁶⁻¹⁸ (DUMAND). It measures a known distribution of ν radiation passing through an object, defining this as a projection χ , and taking the Fourier transform $F(u, v)$ of the projection over the two-dimensional Fourier space (u, v) . The inverse transform determines the densities. It has been described in detail^{12,13,19-21} and, in fact, is directly related to the very long baseline interferometry techniques used in radio astronomy²¹⁻²³. Two source distributions are depicted: a parallel-beam projection generating a planar slice of data F_1 through $F(u, v)$ (Fig. 1a, b), and a fan-beam projection in Fig. 1c, d generating a cylindrical slice $\tilde{F}_1$ of data on $F(u, v)$. Many multiple projections are required to map or 'fill in' the surface $F(u, v)$ in Fig. 1b.

Treated mathematically, the mapping problem was first addressed by Radon²⁴ who derived what has now become known as the Radon transform. Tomography^{10,19,20,25} can be defined as reconstructive imaging by means of the inverse Radon transform technique, its relation to the Fourier transform having been extensively discussed^{19,20,26-29}. Whenever the Radon or Fourier transform is inadequately determined, its inverse cannot be properly defined and the scheme is described as a mathematically ill-posed inverse transform problem²⁰.

The Volkova-Zatsepin Tevatron scheme, also proposed^{30,31}, is illustrated in Fig. 1c, which shows that an infinite number of

fan-beam measurements using an infinite number of detectors or detector positions only maps one cylindrical slice $\tilde{F}_1$ of the Fourier transform for the Earth-fixed Tevatron A. We conclude from Fig. 1 that the geographically fixed Tevatron scheme of Volkova-Zatsepin, and its detector counterpart in Fig. 1d, constitute ill-posed inverse Radon transform problems, and therefore do not work^{1,31} except as X-raying schemes, which have limited use in geophysics because they do not determine the densities. (The mobility referred to in ref. 31 is for geophysical prospecting, not for solution of the inverse Radon transform problem.)

The well-posed inverse Radon transform problem may be realized as follows. The source distribution must be known and measurable, and the neutrino source and detector must both rotate relative to the Earth, mapping an appropriate part of $F(u, v)$. The dashed circle in the $u-v$ plane of Fig. 1c, d can be visualized as a fan-beam 'bubble' in momentum space located beneath the Tevatron A in real space. As A moves geographically, the bubble rotates about the origin of Fourier space and maps $F(u, v)$. Well-posed mapping schemes can be improvised from Fig. 1.

Having a well-posed scheme, the opacity of the Earth must be estimated. It can be determined from seismic models of the Earth's density distribution. Standard models have been described by Press³² and Dziewonski and Anderson³³, supplemented by extensive work on the geochemical composition of the Earth by others³⁴⁻³⁷. We adopt the Dziewonski-Anderson preliminary reference earth model (PREM) here primarily because it states explicitly the radial density functions ρ . It is compatible with the Monte Carlo study of Press³². The results are given in Table 1 and illustrated in Fig. 2. Figure 3a will be used in the derivation.

The PREM density distributions define zones or concentric shells³³. Cords of path length L at angle ψ from the nadir were chosen tangent to each shell at a depth $d = a(1 - \sin \psi)$, where $a = 6,371$ km is the radius of the Earth. By means of a simple coordinate transformation, line integrals were then performed along L to determine the total nucleon mass $M = M(\psi)$ in a 1 cm^2 cylindrical column at angle ψ through the PREM model:

$$M(\psi) = \int \rho(\psi, L) dL \quad (1)$$

(ρ in equation (1) is actually measured in ν -tomography by the inverse Radon transform technique already described.) The total mass was next divided by the mean mass per nucleon $\bar{m}_n$ to

Table 1 Neutrino tomography, from the PREM Earth model

PREM zone (density g cm^{-3})	ψ (deg)	n ($\times 10^{34}$)	L (10^4 km)	$N = n/L$ (10^{24} cm^{-3})	$\kappa = \sigma_{\nu N} N$ (10^{-10} cm^{-1})			$\lambda = \kappa^{-1}$ (Earth radii)			% Attenuation ($1 - I/I_0$) $\times 100$		
					1 TeV	10"	100"	1 TeV	10"	100"	1 TeV	10"	100"
Central core (13.09-12.70)	0.0	0.6600	1.2742	5.1796	0.33	2.3	9.8	48.1	6.7	1.6	4.1	25.7	71.5
Outer core (12.17-9.90)	11.05	0.5362	1.2506	4.2877	0.27	1.9	8.1	58.1	8.1	1.9	3.3	21.4	63.9
Lower mantle (5.57-4.38)	33.11	0.3770	1.0674	3.5511	0.22	1.6	6.7	70.2	9.8	2.3	2.4	15.7	51.3
Transition zone (3.99-3.98)	63.49	0.1774	0.5688	3.1183	0.20	1.4	5.9	79.9	11.2	2.6	1.1	7.7	28.6
Transition zone (3.98-3.72)	64.93	0.1634	0.5398	3.0273	0.19	1.36	5.8	82.3	11.5	2.7	1.0	7.1	26.7
Transition zone (3.54-3.44)	69.59	0.1084	0.4444	2.4385	0.15	1.1	4.6	102.2	14.3	3.4	0.7	4.8	18.6
Low velocity zone (2.69-3.38)	74.90	0.0694	0.3320	2.0900	0.13	0.9	4.0	119.2	16.7	4.0	0.4	3.1	12.4
Crust (2.90)	84.98	0.0180	0.1114	1.6191	0.10	0.73	3.1	153.9	21.5	5.1	0.1	0.8	3.4
Crust (2.60)	86.08	0.0128	0.0874	1.4669	0.09	0.66	2.8	169.9	23.8	5.6	0.08	0.6	2.4
Ocean (1.02)	88.25	0.0024	0.0391	0.6150	0.04	0.3	1.2	405.1	56.7	13.4	0.0	0.1	0.5

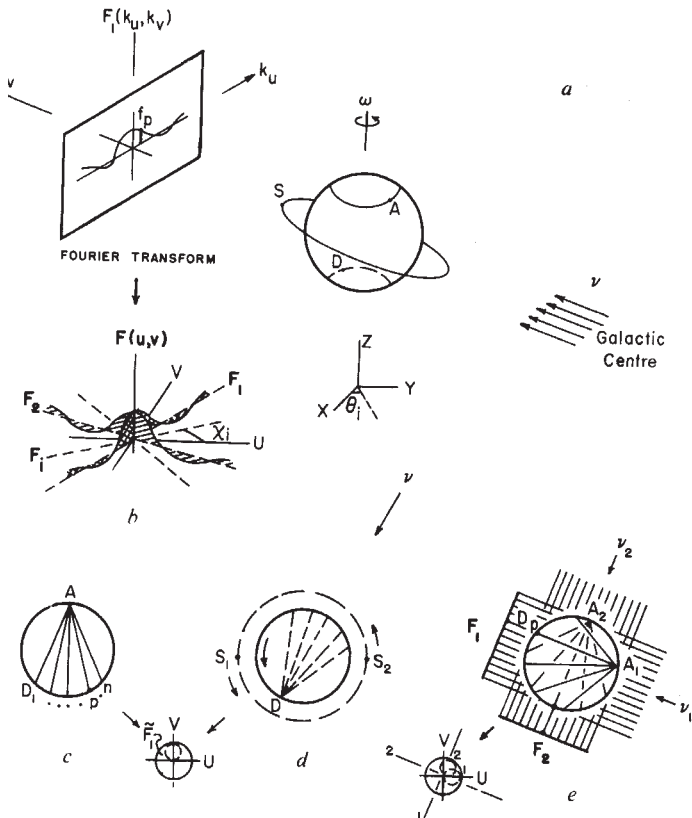


Fig. 1 Neutrino tomography represented as an idealized problem of mapping the Fourier transform $F(u, v)$ of projections of high-energy neutrinos ν through the Earth. *a*, With the Earth fixed ($\omega = 0$) in the inertial frame XYZ a ν -detector S orbiting in the galactic plane could generate one slice $F_1(u, v)$ of the transform in two-dimensional momentum space (k_u, k_v) of Fourier space (u, v). *b*, The actual Earth rotates in the inertial XYZ frame ($\omega \neq 0$) about the Z -axis shown, and for any Right Ascension θ_i , S would generate another slice F_i , gradually mapping $F(u, v)$. *c*, An Earth-fixed fan-beam scanning scheme is shown consisting of an accelerator A , beaming TeV neutrinos through the Earth to a mobile ν -detector D . *c* is mathematically ill-posed; it can generate only one cylindrical slice $\tilde{F}_1$ of points on the surface $F(u, v)$, depicted by the dashed circle (a polar vector of magnitude $a \sin \psi$) in the u - v plane of *c*. *d*, Another ill-posed scheme using an Earth-fixed detector D which scans the Earth's interior while the Earth rotates about the celestial sphere. Schemes *c* and *d* pose acceptable imaging configurations only when A and D are moved. *e*, Part of a well-posed global ring of collaborative Tevatrons, or mobile positions. $\tilde{F}$ is constructed by knowing that $A_1 D_p \rightarrow f_p$ is measured by the same fan or parallel beam. To visualize measuring the density of one point P^* within the Earth, its contour of data on $F(u, v)$ is the smallest cylindrical slice $\tilde{F}^*$ (that is, a smaller dashed circle like that in *c*) osculating with P^* and the centre of the Earth. $\tilde{F}^*$ is measured by moving A on the surface and measuring values of $\tilde{F}$ in *c* where it intersects $\tilde{F}^*$ while $\tilde{F}$ rotates around the u - v origin.

derive the number of nucleons n in the column L . That is, $n = \bar{m}_n^{-1} M$ where $\bar{m}_n^{-1} = 6.029030 \times 10^{23} \text{ g}^{-1}$ is approximately Avogadro's number N_A , and was derived from refs 38, 39 assuming an equal number of protons and neutrons, an average binding energy per mass number of 8.5 MeV and mass numbers of 24 for the mantle and 46 for the core^{36,37}.

Dividing the nucleon number n by the volume of the column which is $L \text{ cm}^3$, we derive the nucleon density N averaged along the cord: $N = n/L \text{ cm}^{-3}$. Given a neutrino-nucleon scattering cross-section $\sigma_{\nu N}$ (an area in cm^2), the neutrino's opacity κ_ν follows dimensionally as the product of $\sigma_{\nu N}$ and N :

$$\kappa_\nu = \sigma_{\nu N} N = \sigma_{\nu N} n/L \text{ cm}^{-1} \quad (2)$$

The neutrino's mean free path is simply the inverse of its opacity: $\lambda_\nu = \kappa_\nu^{-1} \text{ cm}$.

The issue of critical importance in equation (2) is the energy dependence of $\sigma_{\nu N}$. At energies below 1 TeV, experimental groups³⁸⁻⁴¹ have corroborated the assumption that $\sigma_{\nu N}$ increases linearly with neutrino energy E_ν ,

$$\sigma_{\nu N} = 0.67 \times 10^{-38} E_\nu \text{ cm}^2 \quad (3)$$

where E_ν is in units of $\text{GeV} = 10^{-3} \text{ TeV}$. Equation (3) is known as the Bjorken scaling hypothesis^{42,43}. It is shown graphically as the dashed straight line in Fig. 3a, and is predicted by the ordinary four-fermion theory of the weak interaction⁴⁴. It is equivalent to the assumption that $M_W = \infty$, and was used in refs 1 and 31. However, a fundamental tenet of particle physics is that equation (3) cannot be valid at arbitrarily high energies, because it eventually violates what physicists refer to as the unitarity of the scattering matrix^{44,45}. It was shown⁴⁶ that the deep-inelastic cross-section levels off logarithmically at higher energies due to virtual W boson propagator effects (equation (6.4) of ref. 46),

$$\sigma_{\nu N} \sim \log E_\nu \quad (4)$$

instead of increasing linearly as in equation (3).

Theoretical studies of neutrino cross-sections have shown the Bjorken-Paschos effect in equation (4) to be prevalent at tomographic energies (10 – 10^4 TeV). Original concern was with the production of real W bosons⁴⁷⁻⁵⁴ by elastic scattering from target nuclei⁴⁷⁻⁵¹ (coherent) or individual nucleons⁴⁸⁻⁵¹ (incoherent) at small values of the W boson mass ($M_W \sim 5 \text{ GeV}$). As estimates for M_W increased ($M_W \sim 80 \text{ GeV}$), the earlier results⁴⁹ were revised⁵²⁻⁵⁴ for elastic W boson production while interest continued in the deep-inelastic case^{41,52-55} where the target nucleon breaks up into hadronic debris X (for example, $\nu + N \rightarrow \mu + X$). It involved the production of virtual (not real) W bosons^{41,51-54}, and was shown⁵¹⁻⁵⁴ to be the dominant^{52,53,55} effect at the energies considered here.

We adopt the values for $\sigma_{\nu N}$ as those stated by Halprin⁵³ using the quark-parton distributions of Buras and Gaemers^{51,56} for $M_W = 80 \text{ GeV}$ as announced by CERN^{8,9}, with a Bjorken-Paschos logarithmic extension from equation (4). This is shown graphically in Fig. 3a, which is the basis for Figs 2 and 3. (Had M_W been much smaller, $\sigma_{\nu N}$ in Fig. 3a would level off as shown by the dotted curve at a value too low for ν -tomography to work for planetary densities.)

Tomography (Fig. 2) is concerned with the measurement of the transmitted intensity I of neutrinos as compared with the incident intensity I_0 (refs 10, 11). I_0 must be known. These are related to κ and λ by

$$I = I_0 e^{-\kappa L} = I_0 e^{-L/\lambda} \quad (5)$$

as a function of path length L through the Earth. The attenuation is $(1 - I/I_0)$ and is given in Fig. 2 as a percentage for 1, 10 and 100 TeV using the PREM values. The Earth is still virtually transparent at 1 TeV, tomography of the core is possible at 10 TeV and imaging out into the upper mantle is possible at 100 TeV, if we assume a minimum attenuation of 10% to account for measurement uncertainties. Surface tomography³¹ below 100 TeV at PREM densities appears meaningless (except for very dense anomalies there) because the mean free path exceeds 5 Earth radii at that energy (Table 1).

Because tomography cannot be performed without a knowledge of the source distribution I_0 , Tevatron tomography offers the distinct advantage that I_0 can be accurately measured and controlled. On the other hand, we have seen that the inverse Radon transform problem requires an unrealistic, global distribution of Tevatrons and DUMAND-type detectors. We now address the question posed by Fig. 1d of the existence of a tomographic sky, treating ν -tomography as a discipline in ν -astronomy and requiring only the detectors. Neutrino tomography might be feasible as an analysis of the nadir data cuts, using I_0 determined from the zenith data cuts in neutrino astronomy.

Natural sources, predicted in the neutrino sky, are illustrated in Fig. 3, adapted from Margolis, Schramm and Silberberg^{57,58}

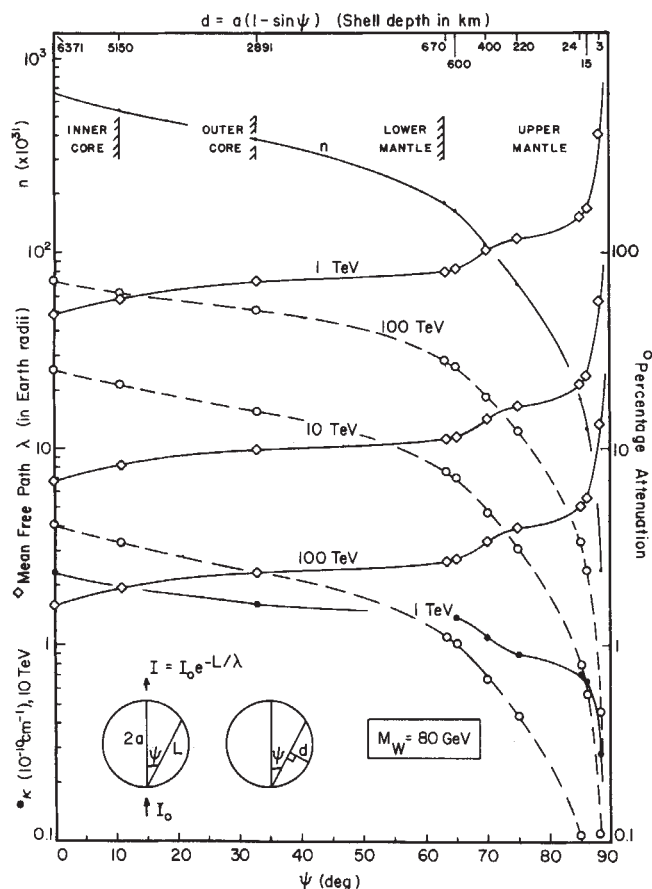


Fig. 2 Opacity of the Earth to neutrinos, derived from the PREM density model of ref. 33. The number of nucleons n in a cylindrical column 1 cm^2 in area along a cord of length L is plotted as a function of the angle ψ from the nadir. From n and L a neutrino opacity κ is derived as a function of neutrino cross-section (shown in Fig. 3a) and therefore energy. The mean free path of the neutrino $\lambda = \kappa^{-1}$ is shown by solid lines, and the corresponding percentage attenuation is given as the dashed lines at the same energies. Tomography 'works' when the attenuation exceeds some threshold value (assumed here as 10%, see ref. 18, p. 235) which accounts for measurement uncertainties. Neutrino oscillations¹⁶⁻¹⁸ have been neglected. (An actual beam of neutrinos from an accelerator diverges, forming a conical frustrum whose volume is approximately $AL/3$, where A is the area of the ν -beam as it leaves the Earth; the number of nucleons n' in the beam's volume is $n' = An/3$. Although n' is $10^{10}/3$ times n for $L = 2a$ and $A = 1 \text{ km}^2$ using a π -meson source in ref. 1, mean nucleon density is unchanged. Tomographic resolution is sacrificed as n' increases for different ν -sources.)

and Stecker⁵⁹. The sensitivities of various detectors are derived for $M_W = 80 \text{ GeV}$, which is essentially an inversion of $\sigma_{\nu N}$ in Fig. 3a to the appropriate flux ϕ . The flux for a 10^9 ton detector at 1 TeV, assuming $M_W = \infty$, is shown as ϕ_1 which calibrated our curve c (Fig. 3), and from which followed curves b and d . We can see from Fig. 3 that a 10^{11} ton DUMAND detector can feasibly measure 100 TeV tomographic neutrinos from the (distended) galactic centre. It seems less probable that a 10^9 ton detector (which measures down to 10 TeV) could differentiate these galactic centre neutrinos from atmospheric, co-rotating neutrinos originating on the opposite side of the Earth and passing through it. (Neutrinos from co-rotating sources, such as the atmosphere and from cosmic rays colliding with the interior, can conceivably be cut from the data using tomographic arguments.) Any other, as yet undiscovered, point source of tomographic neutrinos in the neutrino sky (such as the supernova remnant Cas A or the radio galaxy Cen A, discussed in ref. 57) whose flux lies above the DUMAND detector sensitivity

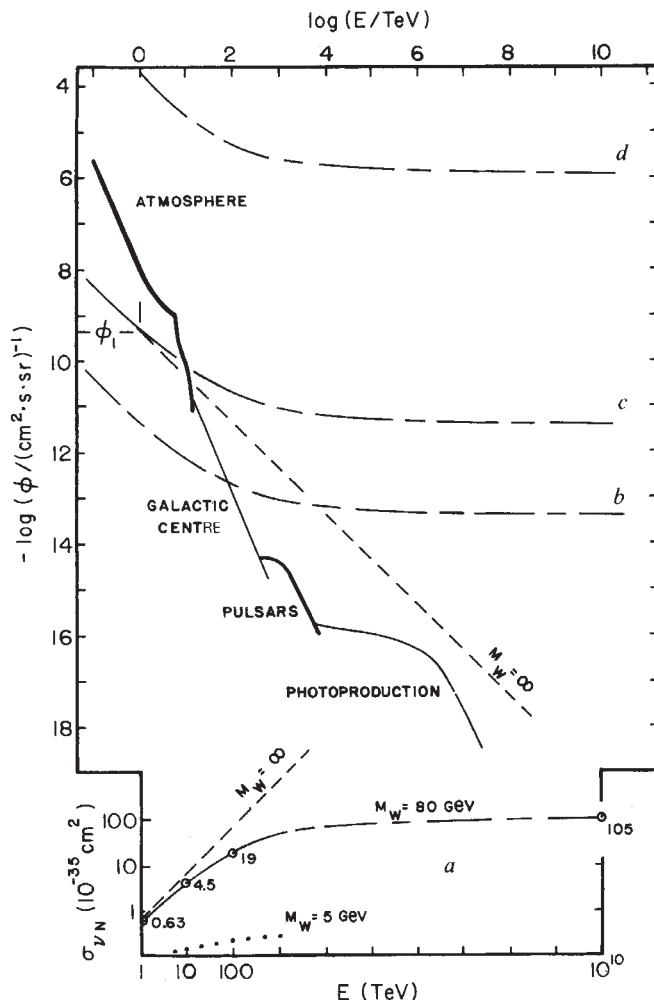


Fig. 3 Natural sources of neutrinos for reconstructive imaging of the Earth's interior density. The neutrino flux ϕ is shown by the solid curves as a function of TeV energy, first compiled in refs 57 and 59. The logarithmic energy scales are equivalent (the upper used in ν -astronomy and the lower in particle physics). The dashed curves are detectors. The flux ϕ_1 at 1 TeV for the 10^9 ton detector is $4.8 \times 10^{-10} \text{ cm}^{-2} \text{ s}^{-1} \text{ sr}^{-1}$. Inset a shows the neutrino-nucleon cross-section $\sigma_{\nu N}$ as a function of TeV energy for $M_W = 80 \text{ GeV}$, and is not a part of the flux diagram. The values indicated at 1, 10 and 100 TeV are taken from ref. 53, while those at 10^3 and 10^4 TeV (35 and 45, respectively) are not shown because the validity of the Buras-Gaemers⁵⁶ assumptions in that energy range and beyond is questionable. For $E_\nu > 10^3$ TeV, the curve is dashed to emphasize that it represents only an extension of the Bjorken-Paschos logarithmic term in the Buras-Gaemers-Halprin cross-section at 1, 10, 100 TeV, and is unknown beyond 10^4 TeV. b – d , Detectors capable of measuring 1 count per day and they follow from $\sigma_{\nu N}$ in a and ν -flux arguments given in refs 57–59. b is 10^{11} tonnes and c is 10^9 tonnes, both of the type considered for DUMAND. d , The flux sensitivity of one orbiting Shuttle external tank (73 trips with 2,157 tonnes of water), as an illustration of the unrealistic effort required for ν -tomography from near-Earth orbit.

threshold in Fig. 3, might function as well for the 72% core attenuation given in Fig. 2 at 100 TeV.

The flux in Fig. 3 is extremely low (1 event per day), and may be inaccurate by magnitudes. Unless an intense source were discovered in the neutrino sky, the galactic centre represents a varying source I_0 at low flux which means the data (for whole-Earth tomography) must be taken over considerable periods of time. It is feasible but unrealistic.

In conclusion, well-posed mapping schemes require impractical distributions of Tevatrons and neutrino detectors, or detectors over unrealistic periods of time. Whole-Earth tomography

seems out of the question (Fig. 2), requiring inordinate energies (>100 TeV) whose neutrino physics is little understood, although the non-invasive measurement of the density at one or more points in the core is less remote. The true significance of neutrino tomography probably lies not in its implementation but in the importance of where it focuses interdisciplinary attention in geophysics³³ and physics⁴⁷⁻⁵⁵: the neutrino cross-sections⁴⁷⁻⁵⁵ (coherent and incoherent) for constituent substances³³⁻³⁷ at tomographic energies ($10-10^4$ TeV), although the neutrinos appear insensitive^{55,52} to the coherent nuclei at these energies (as was assumed here). The density at one point in the core or lower mantle is determinable, and such a study is feasible but impractical by this analysis.

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- Volkova, L. V. & Zatsepin, G. T. *Acad. Sci. U.S.S.R., Bull. Phys. Ser.* **38**, 151-154 (1974).
- Lee, T. D. & Yang, C. N. *Phys. Rev. Lett.* **4**, 307-311 (1960).
- Schwinger, J. *Ann. Phys.* **2**, 407-434 (1957).
- Bahcall, J. N. & Frautschi, S. C. *Phys. Rev.* **136**, B1547-B1552 (1964).
- Salam, A. *Science* **210**, 723-732 (1980).
- Weinberg, S. *Science* **210**, 1212-1218 (1980).
- Glashow, S. *Science* **210**, 1319-1323 (1980).
- Arnsion, G. *et al. Phys. Lett.* **122B**, 103-116 (1983).
- Banner, M. *et al. Phys. Lett.* **122B**, 476-485 (1983).
- Cormack, A. M. *Science* **209**, 1482-1486 (1980).
- Hounsfield, G. N. *Science* **210**, 22-28 (1980).
- Brooks, R. A. & DiChiro, G. *Phys. med. Biol.* **21**, 689-732 (1976).
- Kak, A. C. *Proc. IEEE* **67**, 1245-1272 (1979).
- Wilson, T. L. in *Particles & Fields—1982* (eds Caswell, W. E. & Snow, G. A.) (American Institute of Physics Conf. Proc. No. 98, New York, 1983).
- Donaldson, R. *et al. (eds) Proc. 1982 DPF Summer Study On Elementary Particle Physics & Future Facilities*, Snowmass, Colorado (American Physical Society, New York, 1983).
- Roberts, A. *et al. (eds) Proc. 1978 int. DUMAND (Deep Underwater Muon and Neutrino Detector) Symp.* (Fermilab, Batavia, 1979).
- Stenger, V. J. (ed.) *Proc. 1980 int. DUMAND Symp.* (University of Hawaii, 1981).
- Stenger, V. J., Learned, J. G., Peterson, V. Z. & Roberts, A. in *Neutrino '81* Vol. 2 (eds Cence, R. J. *et al.*) 233-239 (University of Hawaii, 1981).
- Herman, G. T. (ed.) *Image Reconstruction from Projections* (Springer, Berlin, 1979).
- Herman, G. T. & Natterer, F. (eds) *Mathematical Aspects of Computerized Tomography* (Springer, Berlin, 1981).
- Bracewell, R. N. & Riddle, A. C. *Astrophys. J.* **150**, 427-434 (1967).
- Burke, B. F. *Astronautics and Aeronautics* **20**, (10), 44-52 (1982).
- Fomalont, E. *Proc. IEEE* **69**, 1211-1218 (1973).
- Radon, J. *Ber. Verh. sächs. Akad. Wiss.* **69**, 262-277 (1917).
- Cramér, H. & Wold, H. *J. Lond. math. Soc.* **11**, 290-294 (1936).
- Soroko, L. M. *Sov. J. Particles & Nuclei* **12**, 303-319 (1981).
- Herman, G. T. & Naparstek, A. *SIAM J. appl. Math.* **33**, 511-533 (1977).
- Herman, G. T., Lakshminarayanan, A. & Naparstek, A. *Comput. Biol. Med.* **6**, 259-271 (1976).
- Roland, S. W. in *Image Reconstruction from Projections* (ed. Herman, G. T.) 9-80 (Springer, Berlin, 1979).
- Nedialkov, I. P. *Acad. Bulgarian Sci.* **34**, 1495-1498 (1981).
- De Rujula, A., Glashow, S. L., Wilson, R. R. & Charpak, G. *Phys. Rep.* **99**, (6), 341-396 (1983).
- Press, F. *Science* **160**, 1218-1221 (1968).
- Dziewonski, A. M. & Anderson, D. L. *Phys. Earth planet. Inter.* **25**, 297-356 (1981).
- Ringwood, A. E. *Composition and Petrology of the Earth's Mantle* (McGraw-Hill, New York, 1975).
- Knopoff, L. & MacDonald, G. J. F. *Geophys. J.* **1**, 284-297 (1958).
- Birch, F. *Phys. Earth planet. Inter.* **1**, 141-147 (1968).
- Liu, L. *Phys. Earth planet. Inter.* **8**, 56-62 (1974).
- Aguilar-Benitez, M. *et al. Phys. Lett.* **111B**, 1-294 (April 1982).
- Kelley, R. L. *et al. Rev. mod. Phys.* **52**, (2), PII, S1-S286 (1980).
- Barish, B. C. *et al. Phys. Rev. Lett.* **39**, 741-744 (1977).
- de Groot, J. G. H. *et al. Z. Phys.* **C1**, 143-162 (1979).
- Bjorken, J. D. *Phys. Rev.* **179**, 1547-1553 (1969).
- Perkins, D. H. *Rep. Prog. Phys.* **40**, 409-481 (1977).
- Barish, B. C. *et al. Phys. Rev. Lett.* **31**, 180-183 (1973).
- Llewellyn-Smith, C. H. *Phys. Rep.* **3C**, 261-379 (1972).
- Bjorken, J. D. & Paschos, E. A. *Phys. Rev.* **D1**, 3151-3160 (1970).
- von Gehlen, G. *Nuovo Cim.* **30**, 859-877 (1963).
- Chen, H. H. *Nuovo Cim.* **64**, 585-612 (1970).
- Brown, R. W. & Smith, J. *Phys. Rev.* **D3**, 207-222 (1971).
- Brown, R. W., Hobbs, R. H. & Smith, J. *Phys. Rev.* **D4**, 794-813 (1971).
- Brown, R. W. *et al. Phys. Rev.* **D6**, 3273-3292 (1972).
- Halprin, A. & Gaisser, T. in *15th int. Cosmic Ray Conf.* Vol. 6, 265-269 (Bulgarian Academy of Sciences, Plovdiv, 1977).
- Halprin, A. in *Proc. 1978 int. DUMAND Symp.* Vol. 2 (eds Roberts, A. *et al.*) 27-40 (Fermilab, Batavia, 1979).
- Brown, R. W. *et al. in Proc. 1978 int. DUMAND Symp.* Vol. 2 (eds Roberts, A. *et al.*) 13-26 (Fermilab, Batavia, 1979).
- Brown, R. W. & Stecker, F. W. *Phys. Rev.* **D26**, 373-383 (1982).
- Buras, A. & Gaemers, K. *Nucl. Phys.* **B132**, 249-267 (1978).
- Margolis, S. H., Schramm, D. N. & Silberberg, R. *Astrophys. J.* **221**, 990-1002 (1978).
- Eichler, D. *Astrophys. J.* **232**, 106-112 (1979).
- Stecker, F. W. *Astrophys. J.* **228**, 919-927 (1979).

Molecular conformations in surfactant micelles

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For more than half a century, the cornerstone to our understanding of molecular organization in amphiphilic aggregates has been Langmuir's principle of differential solubility¹, according to which the polar heads of amphiphilic molecules dissolve in water whereas the hydrocarbon tails cluster in a core in order to minimize solvent contact²⁻⁵. This view has recently been challenged; much spectroscopic data⁶⁻¹⁷ have been construed¹¹⁻¹⁷ to indicate significant penetration of water into the cores of micelles. Here various models of micellar structure are critically tested against results from NMR and recent small-angle neutron scattering (SANS) experiments. Molecular organization is found to be the consequence of Langmuir's principle and of the packing requirements imposed by steric constraints among chain molecules at interfaces. The evidence is unambiguous that these cores are virtually devoid of internal water.

SANS experiments¹⁸ were performed on micelles consisting of terminally-deuterated lithium dodecyl sulphate molecules (3.672×10^{-2} M) in 0.3 M LiCl solution in D₂O at 35 °C. The advantages of these conditions for the present purposes are that the micelles are nearly spherical and monodisperse¹⁸⁻²⁰. The intensity, $I_D(q)$, of elastically scattered neutrons was measured as a function of wave vector $q = (4\pi/\lambda) \sin(\theta/2)$, where θ is the scattering angle and λ ($=4.75$ or 5.00 Å) is the wavelength of the neutrons. Parallel experiments were performed on protonated micelles, from which $I_H(q)$ was obtained. The scattering intensity is approximately separable into factors $P(q)$, representing intramicellar structure, of interest here, and $S(q)$, representing the positional correlations of particles in solution; that is, $I(q) = m P(q) S(q)$ where m is the number density of micelles²⁰. The difference amplitude, $A(q)$, of the scattering from the chain termini can be calculated from the measured intensities by

$$A(q) = \{[I_H(q)]^{1/2} - [I_D(q)]^{1/2}\} [S(q)]^{-1/2} \quad (1)$$

the procedure for calculating $S(q)$ is presented elsewhere¹⁸⁻²⁰. Values for $A(q)$ thus obtained from our experiments are shown in Fig. 1. The radial density distribution, $N_{Me}(r)$, of termini per unit volume throughout the micellar core can be derived from $A(q)$ (ref. 18)

$$A(q) = m^{1/2} \int_0^\infty 4\pi r^2 b_{Me} N_{Me}(r) \left(\frac{\sin qr}{qr} \right) dr \quad (2)$$

where $b_{Me} = |b_{CH_3} - b_{CD_3}| = 3.12 \times 10^{-12}$ cm is the difference in scattering lengths.

Various models of micellar core structure predict different radial density distributions, $N_{Me}(r)$, of the termini therein. Evaluation of equation (2) according to each model described below yields the set of scattering functions $A(q)$ shown in Fig. 1. To calculate these distributions, it is convenient to consider the hydrocarbon core as being partitioned into discrete concentric shells, numbered $i = 1, 2, 3, \dots, n+1$, towards the centre. Chains are assumed to have $n+1$ segments. The i th shell contains $N_i = (4\pi/3) [3(n+2-i)(n+1-i)+1]$ equivolume sites. Each site contains at most one chain segment. For chain lengths to be independent of configuration, sites must be approximately isodiametric^{21,22}; dodecyl chains are thus most suitably

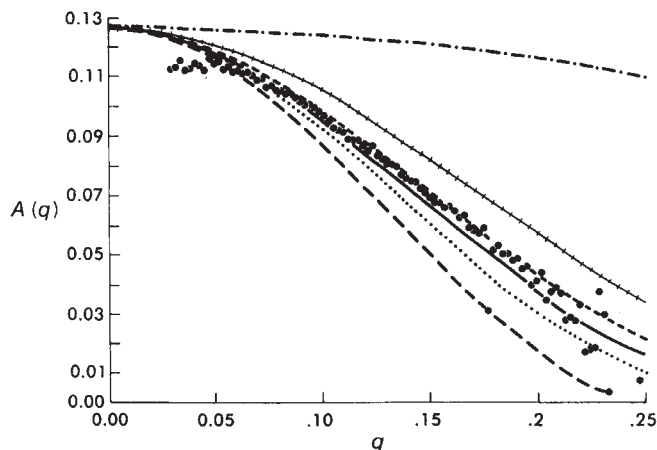


Fig. 1 Small-angle neutron scattering amplitudes, $A(q)$ against wavevector, q . Data from ref. 18, corrected here for interparticle interactions according to equation (1). Predictions (radial shells are numbered $i = 1, 2, 3, 4$, towards the micellar centre) are given by: (1) Radial chain model; $t_i = 0, 0, 0, 1$ (—). (2) Pincushion model; $t_i = 0, 0.563, 0.375, 0.062$ (++++). (3) Interphase model, $\omega = 1$; $t_i = 0.234, 0.414, 0.289, 0.062$ (---). (4) Interphase model, $\omega = 0.5$; $t_i = 0.326, 0.370, 0.241, 0.062$ (—). (5) Interphase model, $\omega \rightarrow 0$ (identical to Cabane model²⁵); $t_i = 0.438, 0.312, 0.188, 0.062$ (·····). (6) Oil droplet model; $t_i = 0.578, 0.297, 0.109, 0.016$ (—).

represented by $n+1 \approx 4$. The end group density may be expressed as $N_{Me}(r) = (J_1/N_i) \cdot [(n+1)/R]^3 \cdot t_i$, where t_i is the fraction of chains whose termini are in layer i , $J_1 = 82$ is the number of chains per micelle, and $R = 18.9 \text{ \AA}$ is the equivalent spherical radius of the micellar core¹⁹.

The following models are considered: (1) The radial chain model. Micellar chains are often depicted as radially aligned and largely all-*trans*^{23,24}. Taken literally, this model would require all termini to occupy the same element of volume at the core centre, in violation of volume exclusion constraints. That this model, which represents the maximum possible radial order and porosity of the core to solvent, is not viable is evident from comparison with the SANS and other results (see Fig. 1 and refs 25, 26).

(2) The pincushion model¹¹ admits of lesser—but significant—water penetration into outer regions of the core. For purposes of calculation, we adopt a version of this model according to which chain ends are distributed as close to the core centre as is consistent with packing constraints. The core centre contains only termini. Sites in the surrounding shell are occupied by the few radially-aligned bonds required to connect to the centre termini and are otherwise filled by chain termini: this shell contains no lateral bonds (that is, those normal to the radius). The next (third) shell is similarly occupied by forward steps to central layers and by the remaining termini. This and surrounding shells may be further occupied by any distribution of lateral and forward segments and solvent molecules with no further bearing on the distribution of termini. Thus, this model represents a class of configurations with water penetration of 20–60% of the outer layers of the core. This model is also in poor accord with the SANS data (Fig. 1).

(3) In the oil droplet model³, the micellar core resembles pure, amorphous, liquid alkane. Accordingly, chain ends are distributed uniformly throughout the core, the number in each shell being proportional to its volume. This model overestimates the methyl density at the solvent interface (see Fig. 1 and ref. 18). Other evidence shows that the chains in micellar cores are more ordered than in liquid alkanes^{1,3,25–30}.

(4) According to the interphase model^{21,22,31,32}, the core is filled exclusively by chain segments which are randomly distributed except as required to preserve chain continuity and satisfy excluded volume constraints. These requirements are taken into account through application of a matrix method^{21,22,31}. The first chain segments connected to head

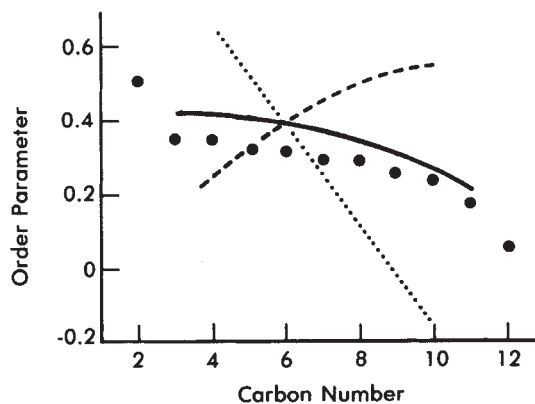


Fig. 2 ^2H -NMR disorder gradient measurements of potassium laurate micelles in the hexagonal phase against chain position from the head group. Data from ref. 27 are multiplied by 2 to correspond to the bond order parameter defined according to the interphase theory^{22,31}: ---, freely flexible chains, $\omega = 1$; —, 'real' chains, $\omega = 0.5$; ·····, rigid molecules, $\omega \rightarrow 0$. The oil droplet model predicts $S = 0$ and the radial chain model $S = 1$ for all segments.

groups are required to occupy the outermost shell of the core; the remaining sites of that shell are filled by laterally bonded segments. Other layers are similarly occupied by bonds which enter shell i from $i-1$, or by lateral bonds in i . Subject to these constraints, chain segment orientation is otherwise random, for freely flexible chains^{21,22}. Real chains are not freely flexible, however. Their stiffness may be taken approximately into account through a statistical weight ω , representing the *a priori* probability of a 'bent' bond pair relative to a co-linear pair in an isolated molecule^{31,32}. With this refinement, freely flexible chains are represented by $\omega = 1$, rigid molecules by $\omega \rightarrow 0$ and real polymethylene chains at $T = 300 \text{ K}$ approximately by $0.4 \leq \omega \leq 0.5$; predictions for these three cases are shown in Fig. 1. Since the interphase model requires complete filling of the core volume, then even for $\omega \rightarrow 0$, most chains are required to have one bend; in this limit, the chain configurations are identical to those predicted by the simple micellar packing model of Cabane²⁵.

The results of the interphase model have been mimicked through an alternative formulation^{33,34} in which intramolecular interactions are treated in the rotational isomeric state approximation³⁵, but in which steric packing constraints are represented through internal pressures which differ in each shell. The latter premise is unsatisfactory inasmuch as the requirement for mechanical equilibrium is thereby violated; these pressures are introduced to simulate packing constraints. The constraints are modelled explicitly, however, by the interphase treatment described above.

Interphase theory predictions are in good agreement with experimental measurements of configurational properties of amphiphilic aggregates^{1,21,22,26,32,36}. We have previously noted that one prediction, however, has been in disagreement with experiment¹; here that issue is resolved. The interphase theory for freely flexible chains predicts that the order should increase towards the chain ends in micelles (see the dashed curve in Fig. 2; see also ref. 22). ^2H -NMR experiments with perdeuterated chains in hexagonal phase micelles²⁷ show the reverse, that there is increasing orientational disorder towards the terminal groups. This closely resembles the 'disorder gradient' in bilayer membranes^{21,37}. Recent refinement of interphase theory^{31,32} shows that orientational order in micelles depends markedly on chain stiffness, neglected in the earlier approximation²² (compare the solid curve in Fig. 2). Intramolecular interactions thus contribute significantly to micellar chain organization whereas for planar bilayers the disorder gradient depends primarily on intermolecular constraints³².

There is broad support for the view that water is largely excluded from the interior of micellar hydrocarbon cores (refs 1, 3, 4, 18, 25 and references therein). This conclusion is corrob-

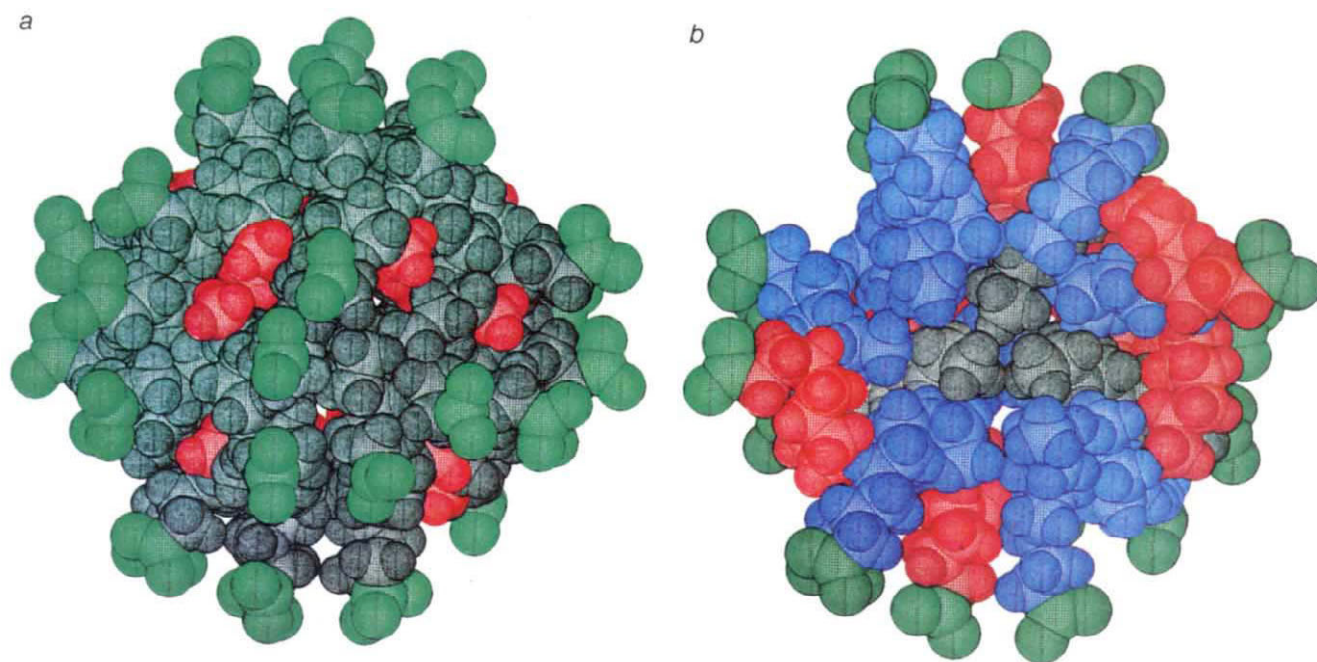


Fig. 3 Representative configuration of a 38-chain spherical decanoate micelle according to the interphase theory^{22,31}. Computer graphics were performed with a modified version of SPACFIL at Molecular Design, Ltd. The micelle was constructed by configuring chains on a spherical lattice, then applying a nonlinear least-squares method to best fit vectors, representing C-C bonds between methylenes, through the appropriate lattice sites. *a*, Outside surface. Head groups in green, methylenes in black and terminal methyls in red. Much hydrocarbon, including some chain ends, is exposed to solvent. *b*, Micellar interior; front hemisphere removed. Red molecules terminate near the surface, blue ones midway to the centre and black ones at the core centre. In this view, chain disorder, distribution of termini and density of packing are evident.

orated by the results of Fig. 1 showing that the differential SANS data are best modelled by dry hydrocarbon core configurations. The strongest support for this view, however, comes from SANS contrast variation measurements of absolute scattering length density^{18,38}. In a series of independent measurements¹⁸, lithium dodecyl sulphate micelles with mean aggregation number of 78 were shown to have a hydrocarbon core volume per molecule ($28,085/78 = 360 \text{ \AA}^3$) equal to that of a pure dodecyl chain³. Moreover, the measured scattering length density of the core ($\rho = -3.8 \times 10^{-7} \text{ \AA}^{-2}$) is exactly equal to that predicted for a dry core consisting exclusively of dodecyl chains ($b_{C12} = -1.37 \times 10^{-12} \text{ cm}$; thus $\rho = -1.37 \times 10^{-4} \text{ \AA}/360 \text{ \AA}^3 = -3.8 \times 10^{-7} \text{ \AA}^{-2}$). These experiments could have readily detected one D_2O molecule per amphiphile in the core. X-ray diffraction experiments³⁹ are more equivocal because they only provide relative densities. The preponderance of support for the alternative view¹¹⁻¹⁷, that there is water penetration, has come from spectroscopic experiments, in which water-insoluble probes in micellar solution have been found to reside in a partly aqueous environment^{6-17,40,41}. The two alternative interpretations of these data are that the probes and water may associate either in the core or at the interface. Recent ring-current shift NMR measurements show that many of these probes reside at the interface⁴⁰⁻⁴². ^{19}F -NMR spectroscopy on perfluorooctanoate micelles shows that the segments of the micellar chains similarly reside in a partly aqueous environment⁴³. This result should not be surprising, however, for nearly 60% of the chain segments in small micelles are at the surface¹. Recent interphase theory calculations predict quantitatively the degree of water contact of each of the chain segments as measured in the ^{19}F -NMR experiments¹; it is the consequence of the high equilibrium incidence of chain segments at the core interface. We conclude that micellar hydrocarbon cores are virtually devoid of internal water, but that substantial hydrocarbon/water contact occurs at the core interface.

Computer graphics representations of the interphase model (Fig. 3*a, b*) show the degree of chain disorder, the distribution

of termini and the extent to which hydrocarbon segments, including chain ends, are exposed to the solvent at the core interface.

The structures and properties of micellar aggregates are thus found to be the consequences of: (1) Langmuir's principle of differential solubility, according to which hydrocarbons are sequestered into a core devoid of water surrounded by the polar heads; (2) the principle that steric forces predominantly determine the structures of condensed phases⁴⁴; and (3) the statistical mechanical principle that degrees of freedom of equal energy (in this case, of the intramolecular chain configurations) are of equal likelihood.

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Note added in proof: In a recent study⁴⁵, it is claimed that micellar chains form 'loops', with termini 200 times more exposed to solvent than internal segments. Those results have no bearing on unperturbed micelles, however, for the SANS results presented here would have detected even a small degree of looping, and showed none. In that study, termini and internal groups were double bonds on probe molecules of significantly different lengths. Neither the locations of those molecules nor their double bonds with respect to any micelles present were known. Double bonds are attracted to water³ and chains of different lengths prefer different phases; few extrinsic probes of micelles are innocuous^{1,4}.

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1. Dill, K. A. in *Surfactants in Solution* Vol. 1 (eds Mittal, K. L. & Lindman, B.) 307-320 (Plenum, New York, 1983).
2. Hartley, G. S. *Aqueous Solutions of Paraffin Chain Salts* (Hermann, Paris, 1936).
3. Tanford, C. *The Hydrophobic Effect* 2nd edn (Wiley-Interscience, New York, 1980).
4. Lindman, B. & Wennerstrom, H. *Topics Curr. Chem.* **87**, 1-81 (1980).
5. Israelachvili, J. N., Marcelja, S. & Horn, R. G. *Q. Rev. Biophys.* **13**, 121-200 (1980).
6. Russell, J. C., Whitten, D. G. & Braun, A. M. *J. Am. chem. Soc.* **103**, 3219-3220 (1981).
7. Russell, J. C. & Whitten, D. G. *J. Am. chem. Soc.* **104**, 5937-5942 (1982).
8. Rodgers, M. A. *J. phys. Chem.* **85**, 3372-3374 (1981).
9. Reed, W., Politi, M. J. & Fendler, J. H. *J. Am. chem. Soc.* **103**, 4591-4593 (1981).
10. Fadnavis, N. & Engberts, J. B. F. *N. J. org. Chem.* **47**, 152-154 (1982).
11. Menger, F. M. *Acc. chem. Res.* **12**, 111-117 (1979).
12. Menger, F. M. & Bonicamp, J. M. *J. Am. chem. Soc.* **103**, 2140-2141 (1981).

13. Menger, F. M. & Boyer, B. J. *J. Am. chem. Soc.* **102**, 5936–5938 (1980).
14. Menger, F. M., Yoshinaga, H., Venkatasubban, K. S. & Das, A. R. *J. org. Chem.* **46**, 415–419 (1981).
15. Turro, N. J. & Okubo, T. *J. Am. chem. Soc.* **103**, 7224–7228 (1981).
16. Martens, F. M. & Verhoeven, J. W. *J. phys. Chem.* **85**, 1773–1777 (1981).
17. Menger, F. M., Jerkunica, J. M. & Johnston, J. C. *J. Am. chem. Soc.* **100**, 4676–4678 (1978).
18. Bendedouch, D., Chen, S. H. & Koehler, W. C. *J. phys. Chem.* **87**, 153–159 (1983).
19. Bendedouch, D., Chen, S. H. & Koehler, W. C. *J. phys. Chem.* **87**, 2621–2628 (1983).
20. Kotlarchyk, M. & Chen, S. H. *J. chem. Phys.* **79**, 2461–2469 (1983).
21. Dill, K. A. & Flory, P. J. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3115–3119 (1980).
22. Dill, K. A. & Flory, P. J. *Proc. natn. Acad. Sci. U.S.A.* **78**, 676–680 (1981).
23. Cantor, C. R. & Schimmel, P. R. in *Biophysical Chemistry* Vol. 3, 1346 (Freeman, San Francisco, 1980).
24. Atkins, P. W. *Physical Chemistry* 2nd edn., 845 (Freeman, San Francisco, 1982).
25. Cabane, B. *J. Phys., Paris* **42**, 847–859 (1981).
26. Dill, K. A. *J. phys. Chem.* **86**, 1498–1500 (1982).
27. Mely, B., Charvolin, J. & Keller, P. *Chem. Phys. Lipids* **15**, 161–173, (1975).
28. Kalyanasundaram, K. & Thomas, J. K. *J. phys. Chem.* **80**, 1462–1473 (1976).
29. Simon, S. A., Stone, W. L. & Busto-Latorre, P. *Biochim. biophys. Acta* **468**, 378–388 (1977).
30. Czarniecki, M. F. & Breslow, R. *J. Am. chem. Soc.* **101**, 3675–3676 (1979).
31. Dill, K. A. & Cantor, R. S. *Macromolecules* **17**, 380–384 (1984).
32. Cantor, R. S. & Dill, K. A. *Macromolecules* **17**, 384–388 (1984).
33. Gruen, D. W. R. in *Surfactants in Solution* Vol. 1 (eds Mittal, K. L. & Lindman, B.) 279–309 (Plenum, New York, 1984).
34. Gruen, D. W. R. *J. Coll. Int. Sci.* **84**, 281–283 (1981).
35. Flory, P. J. *Statistical Mechanics of Chain Molecules* (Wiley-Interscience, New York, 1969).
36. Busico, V. & Vacatello, M. *Molec. Cryst. Liquid Cryst.* **97**, 195–207 (1983).
37. Seelig, J. Q. *Rev. Biophys.* **10**, 353–418 (1977).
38. Cabane, B. in *Surfactants in Solution* Vol. 1 (eds Mittal, K. L. & Lindman, B.) 373–404 (Plenum, New York, 1984).
39. Svens, B. & Rosenholm, B. *J. Coll. Int. Sci.* **44**, 495–504 (1973).
40. Ganesh, K. N., Mitra, P. & Balasubramanian, D. in *Surfactants in Solution* Vol. 1 (eds Mittal, K. L. & Lindman, B.) 599–612 (Plenum, New York, 1984).
41. Ganesh, K. N., Mitra, P. & Balasubramanian, D. *J. phys. Chem.* **86**, 4291–4293 (1982).
42. Zachariasse, K. A. & Kozankiewicz, G. in *Surfactants in Solution* Vol. 1 (eds Mittal, K. L. & Lindman, B.) 565–585 (Plenum, New York, 1984).
43. Muller, N. & Simson, H. *J. phys. Chem.* **75**, 942–945 (1971).
44. Chandler, D., Weeks, J. D. & Andersen, H. C. *Science* **220**, 787–794 (1983).
45. Menger, F. M. & Doll, D. W. *J. Am. chem. Soc.* **106**, 1109–1113 (1984).

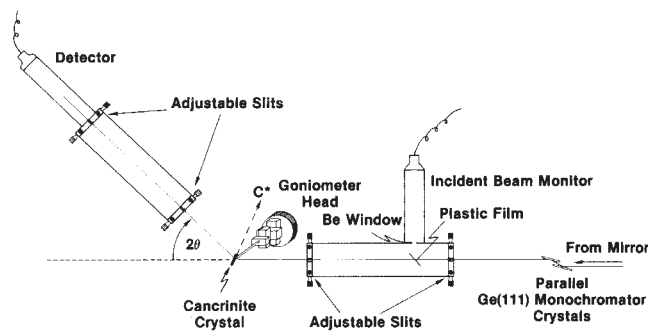


Fig. 1 Schematic diagram of the experimental arrangement. The evacuated boxes housing the adjustable slits and the beam monitor arrangement are shown. Because of the beam polarization, the scattering plane is vertical and the crystal orientation was chosen such that the c^* axis would lie in this plane at $\chi \approx 0^\circ$ (as indicated).

Synchrotron X-ray diffraction from a $800 \mu\text{m}^3$ zeolite microcrystal

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The brightness of synchrotron X-ray sources, which exceeds that available from conventional generators by many orders of magnitude^{1–3}, provides the possibility of making conventional measurements on much smaller samples. We describe here measurements of Bragg diffracted intensities from two microcrystals of cancrinite, a typical zeolite, performed in order to assess the scope of microcrystal diffraction. Our results demonstrate that many materials previously regarded as polycrystalline can now be studied using well established single-crystal methods at synchrotron facilities.

Crystals of basic/nitrate cancrinite^{4–7} were selected from a hydrothermal crystallization batch (F. W. Webster and E. A. D. White, unpublished) that contained specimens ranging in size from several micrometres up to millimetres. Cancrinite is a typical zeolite with an open structure built of atoms of low atomic number [actual composition in this case $\text{Na}_6\text{Al}_6\text{Si}_6\text{O}_{24} \cdot 0.33(\text{NaNO}_3) \cdot 1.22(\text{NaOH}) \cdot 2.2(\text{H}_2\text{O})$] and with some structural imperfection. It therefore provides a near 'worst case' test. One of the larger crystals from the batch had been measured on a conventional CAD-4F diffractometer⁸, enabling us to make a direct correlation between measured intensities and structure factors of known magnitude. The cancrinite structure has hexagonal symmetry and the crystals generally had needle-like morphologies, the long direction coinciding with the unique crystallographic axis. This elongate characteristic allowed the 001 direction to be defined visually in an optical microscope, the selected crystals being too small to align by conventional X-ray photographic techniques. Two crystals of approximate dimensions $35 \times 25 \times 25 \mu\text{m}$ (no. 1) and $20 \times 10 \times 4 \mu\text{m}$ (no. 2) (dimensions estimated by reference to a $12.5\text{-}\mu\text{m}$ eyepiece scale) were selected, glued to the ends of Pyrex glass fibres of $\sim 10 \mu\text{m}$

outside diameter, cut to 1 cm long, mounted on eucentric goniometer arcs and aligned so as to rotate about a direction perpendicular to the long axis.

Measurements were made on the 4-circle Huber diffractometer located at the VII-2 Wiggler line at the Stanford Synchrotron Radiation Laboratory, Stanford⁹, during the tune-up period after the summer shutdown, so that the beam condition was rather poor (see below). On the VII-2 beam line, 4.6 mrad of arc can be viewed by the mirror which focuses the beam at the sample position. After the mirror, a mean incident wavelength of $1.74 \text{ \AA}$ was selected from a pair of parallel, asymmetrically cut Ge(111) crystals. Both incident and diffracted beams were collimated by evacuated adjustable slit systems (Fig. 1). The arcs with the cancrinite crystals were mounted directly onto the diffractometer, the only adjustment made being that of sample height. No particular precautions were taken to minimize the background.

At the chosen wavelength, we had access to two significant 00 l reflections, $l = 2$ and $l = 4$. The configuration was chosen so that the c^* axis would lie in the scattering (vertical) plane (Fig. 1). The crystals were rotated about the spindle axis with the detector set at the 002 position until significant intensity was observed in the counter meter. The reflections were then centred incrementally in each of the circles. In both cases, only minor adjustment of the χ -circle was necessary, demonstrating the effectiveness of the initial optical alignment procedure. Table 1 summarizes the data obtained in radial and rocking-curve scans of the crystals, while scans of the smaller crystal are plotted in Fig. 2. For the larger crystal (no. 1), we also made a scan along 00 l from $l = 1.990$ to 3.015 which indicated that both satellites and the 003 reflection have structure factors of less than 0.9 electrons.

The total energy, E , reflected by a crystal volume δV as it is rotated through the region of a Bragg reflection hkl is (see, for example, ref. 10)

$$E = I_0 \left(\frac{e^2}{4\pi\epsilon_0 mc^2} \right)^2 \frac{\lambda^3}{V^2} \delta V |F_{hkl}|^2 \frac{1}{\sin 2\theta} \frac{1}{\omega}$$

We have used this expression to estimate I_0 , the incident photon flux within the resolution element sampled, from the integrated intensities given in Table 1. The results are given in Table 2. Considering the poor beam conditions, the errors associated with estimating E (Table 2) and the crystal dimensions (as above), the agreement between the three independent estimates is reasonable.

The measured background levels were low. For example, at $h = 0$, $k = 0$, $l = 2.1$ (well away from the central Bragg point), the backgrounds were 2.4 (no. 1) and 0.7 (no. 2) counts s^{-1} , respectively. On the edge of the wings of the Bragg peaks, three halfwidths from the central point, the observed signals were 8.5

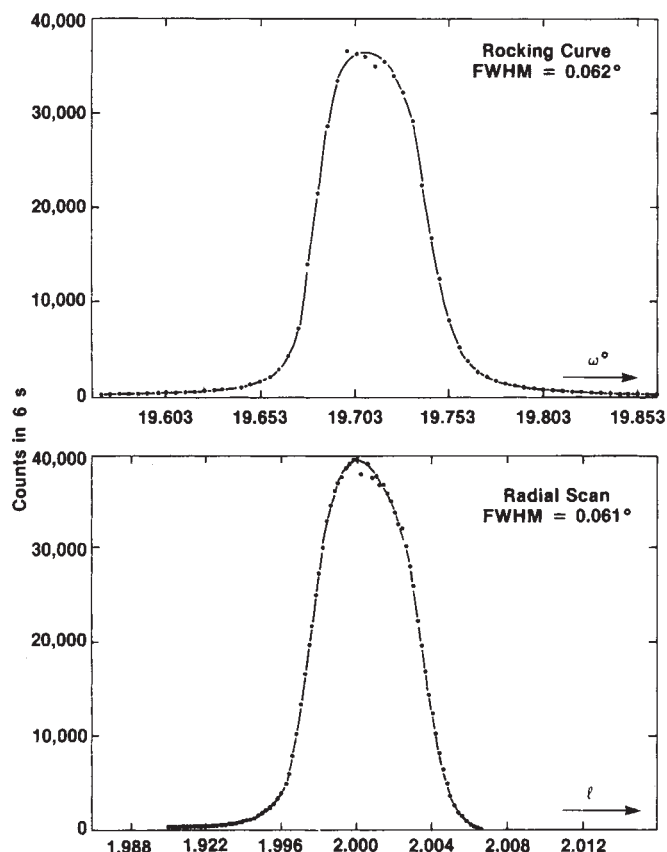


Fig. 2 Scans through the 002 reflection from the 800- μm^3 cancrinite crystal (no. 2). The upper, rocking curve indicates the mosaic spread of the crystal.

(no. 1) and 6 (no. 2) counts s^{-1} . We believe that the background levels could be further reduced by improved crystal mountings, optimized collimation and use of evacuated or helium flight paths. (Background rates as low as 0.1 counts s^{-1} have been achieved in similar experiments on this beam line.)

The size of the smallest measurable crystal will depend on the structure factor (SF) and the counting time allowed to reach a specified signal-to-noise ratio. We will adopt, for illustrative purposes, a very weak SF of 10 electrons, and 100s to achieve

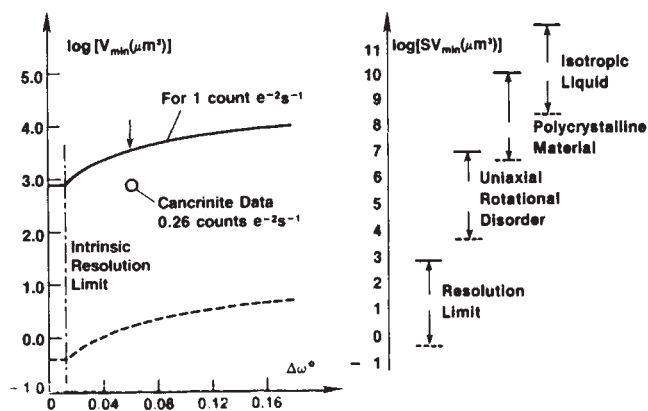


Fig. 3 The minimum crystal volume, $V_{\min}$, that is required to provide a peak count rate of 1 count per electron² per s plotted against the rocking curve width or crystal mosaicity, $\Delta\omega^\circ$. The 800- μm^3 cancrinite crystal provided 0.26 counts per electron² per s (marked). The upper (solid) lines refer to the conditions under which the present measurements were made. The lower (dashed) lines represent the performance expected if an anticipated gain in flux of 2×10^3 is realized (see text). Extrapolation of these curves to $\Delta\omega = 180^\circ$ (corresponding to uniaxial rotational disorder), to peak broadening in the second dimension (representing a random collection of crystallites or 'powder' sample), and finally to peak broadening in 2θ space permit the estimation of the minimum sample volumes ($SV_{\min}$) required to provide 1 count per electron² per s at peak from other types of sample (right-hand portion of figure).

1% statistical accuracy at the peak position. Since in 100 s the background (at 0.1 counts s^{-1}) is expected to be only 10 counts, it can be ignored but larger background rates can obviously increase the minimum measurable volume. In our experiment we recorded equivalent to 0.26 counts per electron² per s or one-quarter of the required accuracy. A complete data set of high precision could thus have been obtained from a $3 \times 10^3 \mu\text{m}^3$ crystal in the conditions of the present experiment.

In optimized beam line conditions, use of a shorter X-ray wavelength and the new Wiggler beam line, one would obtain an enhancement of flux of 2×10^3 – 2×10^4 , with a concomitant reduction in the minimum measurable crystal volume. Thus, in these optimum conditions, complete high-precision data sets could be measured from crystals having volumes less than $1 \mu\text{m}^3$.

It is the brightness of the synchrotron source, the incident

Table 1 Data from radial and rocking-curve scans of crystals

Scan no.	Crystal	Reflection	Type	Background*	Peak*	Intensity*	FWHM (deg)
1	1	002	$\omega - 2\theta$	3	1.1×10^5	8.0×10^6	0.0686
3	1	002	ω	3	1.1×10^5	1.8×10^6	0.0687
5	1	004	$\omega - 2\theta$	3	7.2×10^3	7.8×10^4	0.1412
6	1	004	ω	3	7.2×10^3	1.2×10^5	0.0736
8	2	002	$\omega - 2\theta$	4	6.9×10^3	2.1×10^5	0.0605
9	2	002	ω	4	6.2×10^3	8.4×10^4	0.0620

* Normalized to counts s^{-1} .

Table 2 Estimation of total energy E and incident photon flux I_0 for cancrinite crystals

Crystal no.	Crystal volume	Reflection	F_{hkl}	ω (rad s^{-1})	E	I_0
1	$13,000 \times 10^{-18}$	002	165	2.424×10^{-5}	9.55×10^6	5.24×10^{15}
1	$13,000 \times 10^{-18}$	004	105	1.897×10^{-5}	1.53×10^6	2.54×10^{15}
2	800×10^{-18}	002	165	1.505×10^{-5}	6.36×10^5	3.53×10^{15}

Cancrinite unit cell, $a = 12.69$, $c = 5.16 \text{ \AA}$, $V = 727 \text{ \AA}^3$, space group $P6_3$. The structure factor in electrons, F_{hkl} , was taken to be that measured from a much larger crystal of the same composition on a CAD-4F diffractometer⁸. E is calculated as $I_\omega \cdot f_a$, where I_ω is the integrated intensity recorded in the ω -scan (normalized to the same beam current as the first scan) and f_a is an estimated correction for the diffracted beam collimation in 2θ space (defined as the ratio of the total area of the gaussian of halfwidth FWHM (Table 1) to the area of the gaussian encompassed by the collimation).

photon flux per resolution element, that is the key parameter in this application. At the optimum, the sample mosaic width would match the intrinsically high resolution of the source, but for the crystals studied here, which we believe to be fairly typical, the mosaic width (0.062°) is four times greater than the inherent beam divergence (0.014°). The narrowing in the peak width that would accompany an improvement in crystal quality would enhance the peak count rate and hence further reduce the minimum required sample volume (Fig. 3). To push the technique to its size limit, crystals with a high degree of perfection will be required, although in this size regime one might expect that the smaller the crystal size, the greater the likelihood of crystal perfection.

In many fields great importance is attached to structural characterization and to proper interpretation of the interplay

between structure and properties. Although single-crystal diffraction data are generally required for structure solution and detailed refinement, many materials do not occur in suitable single-crystal form. This has prompted widespread interest in the development of techniques for using powder data in optimizing structures that are already approximately known¹¹. With powder data, particle-size broadening effects generally become apparent for average particle dimensions less than $0.2\ \mu\text{m}$. Our present results indicate that polycrystalline samples in which such effects are not observed can in many cases be regarded as large selections of possible candidates for structure solution and analysis using single-crystal techniques at synchrotron facilities. *Note added in proof:* Bachmann *et al.*¹² recently described measurements on a CaF_2 microcrystal at DESY. Their conclusions are similar to those presented here.

Received 28 September 1983; accepted 6 February 1984.

1. Winick, H. & Doniach, S. (eds) *Synchrotron Radiation Research* (Plenum, New York, 1980).
2. Rowe, E. M. *Phys. Today* 28–37 (1981).
3. Sparks, C. J. *Phys. Today* 40–49 (1981).
4. Pauling, L. *Proc. natn. Acad. Sci. U.S.A.* **16**, 453–459 (1930).
5. Jarchow, O. *Z. Kristallogr.* **122**, 407–422 (1965).
6. Barrer, R. M., Cole, J. F. & Villiger, H. *J. chem. Soc.*, 1523–1531 (1970).
7. Bresciani Pahor, N., Calligaris, M., Nardin, G. & Randaccio, L. *Acta crystallogr.* **B38**, 893–895 (1982).

8. Leonowicz, M. E. & Vaughan, D. E. W. (in preparation).
9. Stanford Synchrotron Radiation Laboratory *Proposal Guidelines and General Information* (SSRL, Stanford, California, 1982).
10. Woolfson, M. M. *An Introduction to X-Ray Crystallography* (Cambridge University Press, 1970).
11. Block, S. & Hubbard, C. R. (eds) *Accuracy in Powder Diffraction* (NBS spec. Publ. 567, National Bureau of Standards, Washington DC (1980).
12. Bachmann, R. *et al. Angew. Chem. int. Edn* **22**, 1011–1012 (1983).

Propagated temperature changes during onset and recovery of the 1982–83 El Niño

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El Niño is the appearance of anomalously warm sea surface temperatures along the coast of Peru. El Niño is also associated with changes of sea level and thermocline depth which are linked to a large-scale interaction of the global atmosphere and tropical ocean^{1–4}. Linear wind driven theory suggests that Kelvin waves are responsible for the El Niño thermocline displacements³, but in model systems it is difficult to relate thermocline changes induced by Kelvin waves to the observed changes in sea surface temperature⁵. We have now examined daily sea surface temperatures along the coast of Peru and subsurface temperature records from the Galapagos and 10°S and 79°W (Fig. 1) made during onset and recovery of the 1982–83 El Niño⁶. Our data show that the onset of the anomalous warming in this region propagated at speeds slower than Kelvin wave speeds. The speed of travel calculated from changes in temperature from the Galapagos to the coast of South America and then poleward along the coast is extremely coherent, and during onset appears to be related to advective processes. The rapid decrease in sea surface temperature associated with recovery from the 1982–83 El Niño was propagated at much higher speeds, very suggestive of Kelvin wave dynamics.

In 1894 Eguiguren⁷ noted that there was a correlation between the rainy years in northern Peru and warm ocean temperatures associated with the years in which the El Niño current was of unusual strength. Half a century later Bjerknes⁸ linked these episodes of warm sea surface temperatures (SSTs) to large-scale meteorological events associated with the Southern Oscillation^{1–4}. Recently Wyrtki² has argued that El Niño is a dynamic response of the entire Pacific basin to changes in large-scale wind forcing; the response of the ocean includes changes in sea level, thermocline depth and horizontal advection.

Observations have shown that changes in sea level propagate rapidly from the western Pacific to the east along the Equator and then poleward along the coast of Peru^{9,10}. Linear wind driven theory suggests that these changes result from packets of Kelvin waves that are excited by anomalies in the trade wind field in the western and central Pacific³. Theory also suggests that Kelvin waves are responsible for the El Niño thermocline displacements. Analysis of the daily temperature changes in 1982 and 1983 contribute to a description of the dynamic characteristics of El Niño and provide an interesting complement to the theory.

Temperature observations made from piers at Paita (5°S), Lobos de Afuera island (7°S), Pimentel (7°S), Supe (11°S) and Callao (12°S) are shown in Fig. 2. The Paita measurements are made at 0800 h daily while the others are averages of measurements made at 0600, 1200 and 1800 h daily. The Paita, Lobos de Afuera and Callao time series (Fig. 2a) show that the initiation of the temperature rise at these locations occurred on 23 September, 29 September and 18 October respectively. The speed of propagation of these temperature rises, assuming straight trajectories is $43.6\ \text{cm s}^{-1}$ between Paita and Lobos de Afuera and $42.3\ \text{cm s}^{-1}$ between Lobos and Callao (Table 1). Smith¹¹ reported that temperature measurements from a depth of 100 m on a current meter array located over the 150 m isobath

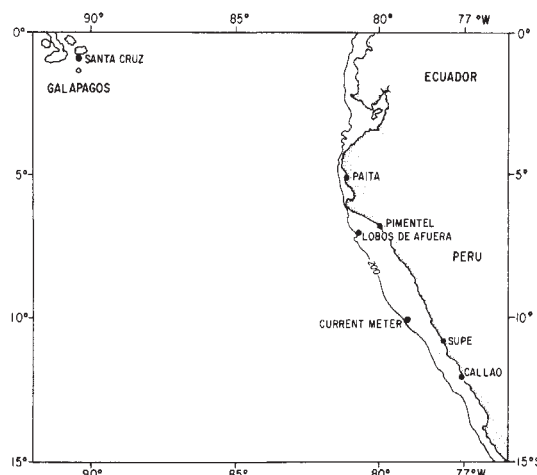


Fig. 1 Chart of the eastern equatorial Pacific showing the locations that provided observations for this article. The 200 fathom contour is included to show the position of the shelf break.

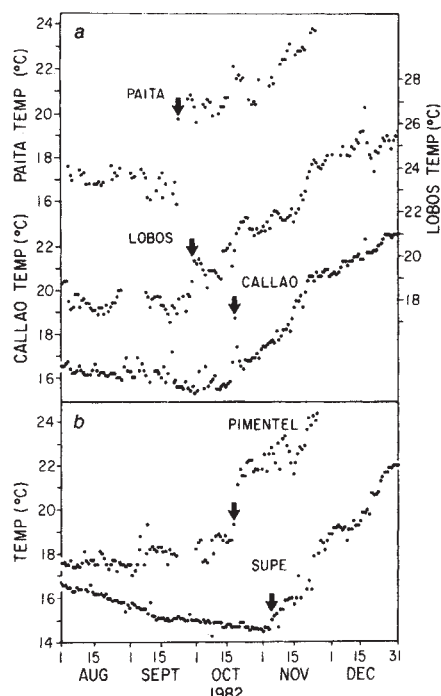


Fig. 2 Daily sea surface temperature observations made from piers at *a*, Paita (5° S), Lobos de Afuera (7° S) and Callao (12° S); and *b*, Pimentel (7° S) and Supe (11° S) during onset of the 1982–83 El Niño. Arrows indicate beginning of the temperature rise used to calculate propagation speeds.

at 10° S showed the beginning of a change in temperature on 7 October. The temperature rise at the surface at a station offshore of Paita which is occupied tri-weekly over the 100 m isobath was first detected on 22 September (no observation made on 21 September). The propagating speed between the Paita offshore station and the 100 m current meter at 10° S is either 43.9 or 46.8 cm s⁻¹. Interestingly the poleward current at 100 m increased in speed from 19 to 36 cm s⁻¹ between 6 and 7 October¹¹, a velocity similar to our calculations of the speed at which the onset of the temperature increase propagated.

Figure 3 shows the temperature time series from the Paita offshore station at 5° S and 81°17' W and a parallel station maintained at Santa Cruz island at 0°47' S and 90°17' W in the Galapagos. The beginning of the temperature rise at 60 m in the Galapagos occurs on 23 August, in agreement with other reports from the islands that warming there began in mid August¹². Assuming a straight line path directly from Santa Cruz to Paita (Fig. 1), the speed of travel of the temperature rise is 42.4 cm s⁻¹; assuming a maximum distance path along the Equator to the coast and then poleward down the coast the speed of travel is 60 cm s⁻¹, a speed used to describe the eastward propagation of the equatorial zonal SST gradient¹³.

A second feature of the SST records from the Peru coast is that the increase in temperature at Pimentel (7° S) and Supe (11° S) (Fig. 2*b*) lag the other coastal stations. The calculated speed of travel from Lobos de Afuera to Pimentel (Fig. 1) is

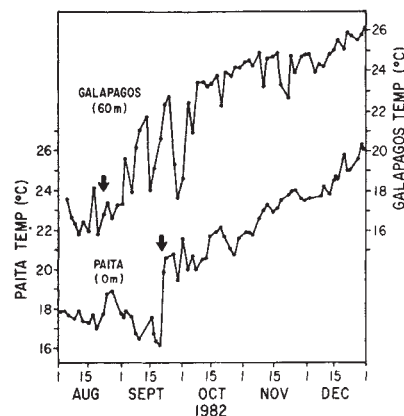


Fig. 3 Tri-weekly temperature observations from 60 m at Paita (5°05' S, 81°16' W) and Puerto Ayora, Santa Cruz Island, Galapagos Islands (0°47' S, 90°17' W). These locations are over the 100 m isobath. Arrows indicate beginning of the temperature rise used to calculate propagation speeds.

5.1 cm s⁻¹. Mean onshore currents of 5.0 cm s⁻¹ have been measured on the coast of Peru¹⁴; this velocity is surprisingly close to the calculated speed for the cross-shelf travel from Lobos de Afuera to Pimentel. Upwelling favourable winds along the coast of Peru remained strong throughout December 1982¹¹, so it is appropriate to assume that a typical cross-shelf upwelling circulation was maintained. Additional evidence for cross-shelf propagation of the temperature changes comes from the Paita offshore station which is 7 km from the coast. At the station the rise led the rise at the Paita pier; however, the tri-weekly sampling does not permit a precise calculation of travel speed over this short distance.

The lag in the temperature rise at Supe (11° S) is difficult to account for. If we assume that a route directly across the shelf from the 200 fathom contour (Fig. 1) then the speed of travel of the rise is calculated to be 4.6 cm s⁻¹. However, there is no reason to restrict the poleward undercurrent to the shelf break as observations during 1977¹⁵ showed the undercurrent was on the shelf at 10° S and 12° S. The lag observed at Supe suggests that the local circulation must have shielded this location.

The mean SST field for this region in September¹⁶ shows that there is a relatively small offshore gradient of SST (17 to 19° C in 100 km) so anomalous onshore surface transport would not result in a significant temperature signal, but there is a strong (17 to 25° C in 100 km) gradient in the alongshore dimension equatorward of Paita. The September 1982 rise in temperature at Paita shows an increase in temperature in a period of a few weeks which is similar to the mean alongshore gradient in that region. Large-scale horizontal temperature distributions¹⁷ for the latter part of 1982 do not show significant changes until November, well after the temperature increases were observed at the shore stations. Our interpretation of this large-scale temperature increase in November 1982 is that it results from movement of the equatorial front southward; temperature, salinity and nutrient observations¹⁸ support this interpretation.

The speed and spatial extent of the decrease in SST and subsurface temperatures associated with recovery from the

Table 1 Travel speeds of the appearance of the anomalous warming between locations shown in Fig. 1

From	On	To	On	Distance (km)	Time (days)	Speed (km day ⁻¹)	Speed (cm s ⁻¹)
Paita pier	23 September	Lobos pier	29 September	226	6	37.7	43.6
Lobos pier	29 September	Callao pier	18 October	695	19	36.5	42.3
Paita offshore	21 September	Current meter (100 m)	7 October	607	16	37.9	43.9
	22 September		7 October	607	15	40.4	46.8
Santa Cruz (60 m)	23 August	Paita offshore	22 September	1,100	30	36.7	42.4
Lobos pier	29 September	Pimentel pier	18 October	83	19	4.4	5.1

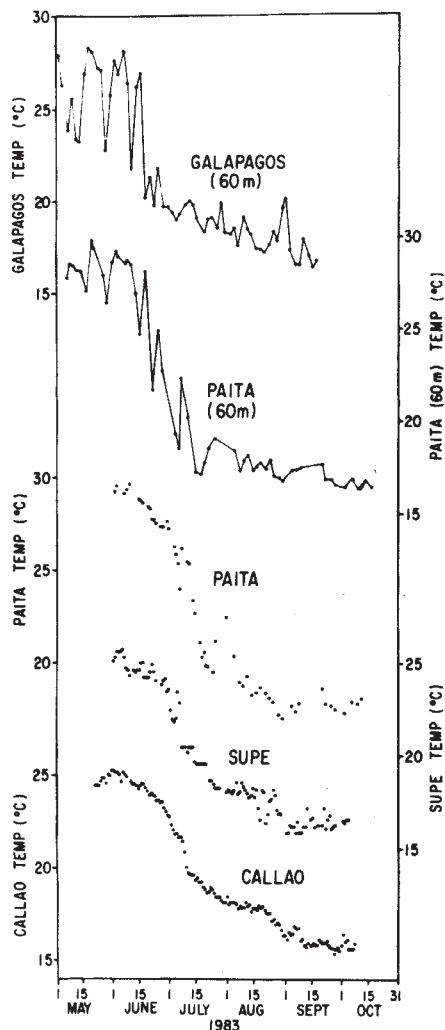


Fig. 4 Tri-weekly temperature observations from 60 m at Paita (5°05' S, 81°16' W) and Puerto Ayora, Santa Cruz Island, Galapagos Islands (0°47' S, 90°17' W) and daily sea surface temperature observations made from piers at Paita (5° S), Supe (11° S), and Callao (12° S) during recovery of the 1982-83 El Niño.

1982-83 El Niño are impressive. Figure 4 shows time series of temperatures from Galapagos (60 m), Paita (60 m), Paita, Supe and Callao during the recovery. Temperatures at 60 m in the Galapagos and Paita and SST from Paita, Supe and Callao drop almost simultaneously. From Fig. 4 it appears that recovery at 60 m in the Galapagos leads the Peru coast recovery and that recovery at 60 m at Paita leads the surface recovery at Paita. From 15 to 17 June there is a 6°C temperature drop at 60 m at the Galapagos, and a similar drop (6°C) is observed at the Paita 60 m station between the 17 and 22 June. Assuming a minimum distance path a propagating speed of 254 cm s^{-1} is calculated; assuming a maximum distance path following the coast and Equator the speed is 345 cm s^{-1} . This velocity domain is suggestive of Kelvin wave dynamics.

The 1982-83 El Niño differed from the canonical event described by Rasmusson and Carpenter⁴ in that onset was some 6 months later in the year, so that during 1982 the anomalies in temperature along the coast of Peru appeared in September as opposed to February; this is important because the temperature fields off the Peru coast during September and February¹⁶ are very different. The remotely forced perturbation in 1982 was, therefore, 'received' by a different thermal regime than the canonical event. Analysis of the day-to-day temperatures changes from previous El Niño events may help elucidate the mechanisms responsible for El Niño SST anomalies.

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1. Philander, S. G. H. *Nature* **302**, 295-301 (1983).
2. Wyrtki, K. *Mar. tech. Soc. J.* **16**, 3-10 (1982).
3. Cane, M. A. *Science* **222**, 1189-1195 (1983).
4. Rasmusson, E. M. & Carpenter, T. H. *Mon. Weath. Rev.* **10**, 354-384 (1982).
5. Busalacchi, A. J., Takeuchi, K. & O'Brien, J. J. *J. geophys. Res.* **88**, 7551-7562 (1983).
6. Gill, A. E. & Rasmusson, E. M. *Nature* **306**, 229-234 (1983).
7. Eguiguren, V. *Bol. Soc. Geogr. Lima* **4**, 241-258 (1894).
8. Bjerknes, J. *Tellus* **18**, 820-829 (1966).
9. Wyrtki, K. *Trop. Ocean-Atmos. Newslett.* **16**, 6-7 (1983).
10. Enfield, D. B. & Allen, J. S. *J. phys. Oceanogr.* **10**, 557-578 (1980).
11. Smith, R. L. *Science* **221**, 1397-1399 (1983).
12. Hayes, S. P. *Trop. Ocean-Atmos. Newslett.* **16**, 17-18 (1983).
13. Harrison, D. E. & Schopf, P. S. *Trop. Ocean-Atmos. Newslett.* **21**, 19-21 (1983).
14. Barber, R. T. & Smith, R. L. *Analysis of Marine Ecosystems* (ed. Longhurst, A. R.) (Academic, New York, 1982).
15. Brockmann, C., Fahrbach, E., Huyer, A. & Smith, R. L. *Deep-Sea Res.* **27**, 847-856 (1980).
16. Zuta, S. & Urquiza, W. *Bol. Inst. mar. Peru* **2**, 460-519 (1972).
17. Auer, S. (ed.) *Oceanogr. Mon. Summ.* **11** (US Department of Commerce, 1982).
18. Barber, R. T. & Chavez, F. P. *Science* **222**, 1203-1210 (1983).

A wetter climate in eastern Sudan 2,000 years ago?

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Within the past 15 years accurate radiocarbon dating and plant microfossil analysis of tropical lake sediments in Africa and elsewhere have revealed that the early Holocene (11-7 kyr) was generally wetter than today throughout the tropics¹⁻⁴, in contrast to last glacial maximum (18 ± 2 kyr), which was cool, dry and windy^{5,6}. Desiccation beginning ~4.5 kyr ago forced Neolithic herders to abandon previously habitable Old World deserts⁷⁻⁹. Less well known is the climate of proto-historic times (~2 kyr ago), when iron-smelting became important in Africa¹⁰. Here we present the first evidence that the Red Sea Hills were somewhat wetter at this time and suggest that this was also true of much of northern Africa.

Prompted by an early report of sub-fossil land and pond snails at Erkowit¹¹ in the arid Red Sea Hills of Sudan, we have collected, identified and dated freshwater molluscs from the alluvial clays which line the main valley near the original site. The mollusc and ostracod assemblage in all our samples is consistent with perennial stream flow some 2,000 years ago in an area now devoid of permanent rivers. The Erkowit plateau lies at 18°5' N, 37°10' E in the Red Sea Hills of eastern Sudan (Fig. 1). It rises over 1,200 m above the Red Sea, from which come frequent winter mists that help sustain sporadic *Euphorbia candelabra* and other evergreen Ethiopian upland plants^{12,13}. Annual rainfall at Erkowit is ~300 mm, with twin peaks in January and August^{14,15}. The surrounding country is much drier¹⁵.

Up to 6 m of shell-bearing, alluvial clays are exposed in ephemeral stream channels like Khor Harasab where they overlie coarse angular gravels (Fig. 2). In nearby tributary gullies the clays sit unconformably on Basement Complex bedrock. Mantling the clays are sands up to 2 m thick, which are still being laid down. Over-grazing has led to gully erosion since the 1920s¹¹, with exposure of the fossiliferous clays.

In addition to the freshwater molluscs *Melanoides tuberculata* and *Bulinus truncatus*, both of which are common in the White Nile, Tothill¹¹ collected two other species of aquatic molluscs from the Erkowit clays (Table 1a). We have identified five species of aquatic snails scattered through the clays (Fig. 2 and Table 1b), but no land snails, in contrast to Tothill, who collected further from the valley bottom¹¹. The ostracod assemblage

found in association with the freshwater mollusc assemblage (Fig. 2) indicates that the clays were laid down in permanent water.

We found shells scattered through the upper 4 m of alluvium, with rather more *Melanoides* and *Biomphalaria* near the top and bottom of the deposit (Fig. 2). The two ^{14}C dates were

from these two levels, and do not differ significantly in age at one standard deviation. After cleaning in an ultrasonic bath and etching with dilute hydrochloric acid, SUA-1794 was counted for 1,760 min, and SUA-1795 for 5,000 min. Only *Melanoides* shells were dated. Each sample was tested by X-ray diffraction for recrystallization, using known proportions of powdered calcite and aragonite as standards. As a further check, we also tested shells of *Melanoides* collected alive from the lower White Nile and from Lake Lyadu in the southern Afar Rift. Despite the unavoidably small samples used for dating, we consider the ages reliable. The lower shell bed dates to $1,710 \pm 160$ yr BP (SUA-1794), the upper to $1,900 \pm 110$ yr BP (SUA-1795).

Of our ostracod assemblage, most species of *Cyprinotus* and *Darwinula* live in fresh water, but some are found in oligohaline to mesohaline water; *Canodopsis* is a freshwater form; and most *Ilyocypris* species are mud-dwellers¹⁶.

Living non-marine charophytes inhabit fresh and brackish water¹⁷. They usually grow submerged in fresh standing water upon a muddy or sandy bottom, and often form extensive subaquatic meadows that may extend to depths of at least 10 m ¹⁸.

Of the molluscs, *Lymnaea* produces gelatinous eggs which dry up when removed from water. Adults can move outside water, but they are essentially aquatic as reproduction is impossible except in water. Modern habitats range from muddy or stagnant ponds and marshes to shallow, fast-flowing streams and lakes^{20,21}. *Melanoides* is a wholly aquatic river or lake bottom species²¹⁻²⁴, but can survive in very shallow brackish water, albeit in dwarfed form, as in the eastern Afar desert. *Lentorhis junodi* lives in slow-flowing streams and marshes²⁴. *Gyraulus connollyi* occurs today in stony or vegetated streams in the temperate uplands of southern Africa²⁴. *Biomphalaria pfeifferi* inhabits stony and vegetated streams²⁴, seems intolerant of very high temperatures^{25,26} and, unlike *B. sudanica*^{27,28} is not found living in small seasonal pools or large swamps.

From the fossil evidence we infer that there was sufficient water on the Erkowit plateau towards 1,700–1,900 yr BP to sustain permanent stream flow in a landscape more densely vegetated than today, with flat-floored swampy meadows in the valley bottoms flanking mixed-load streams that were actively aggrading their channels with sandy or silty muds. More enduring rains during both winter and summer may have raised groundwater levels to the point where perennial base flow became possible.

Was the wetter climate at Erkowit a purely local phenomenon? We think not, as several independent lines of

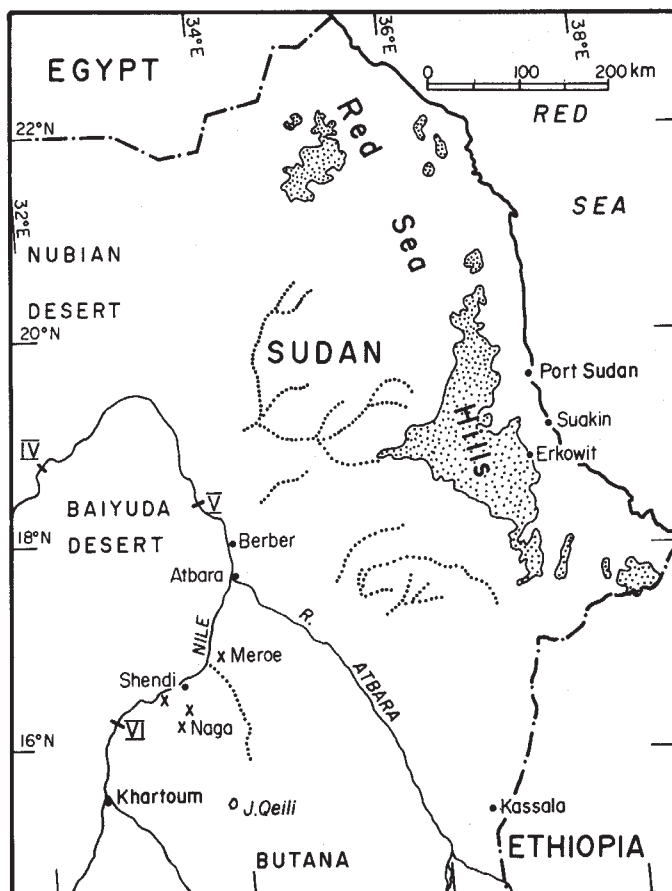


Fig. 1 Map of the northeastern Sudan showing location of Erkowit at the eastern margin of the Red Sea Hills. Crosses denote Meroitic sites (~2,000 yr BP), dotted lines are major ephemeral stream channels; land above 1,000 m is dotted; Roman numerals are Nile cataracts.

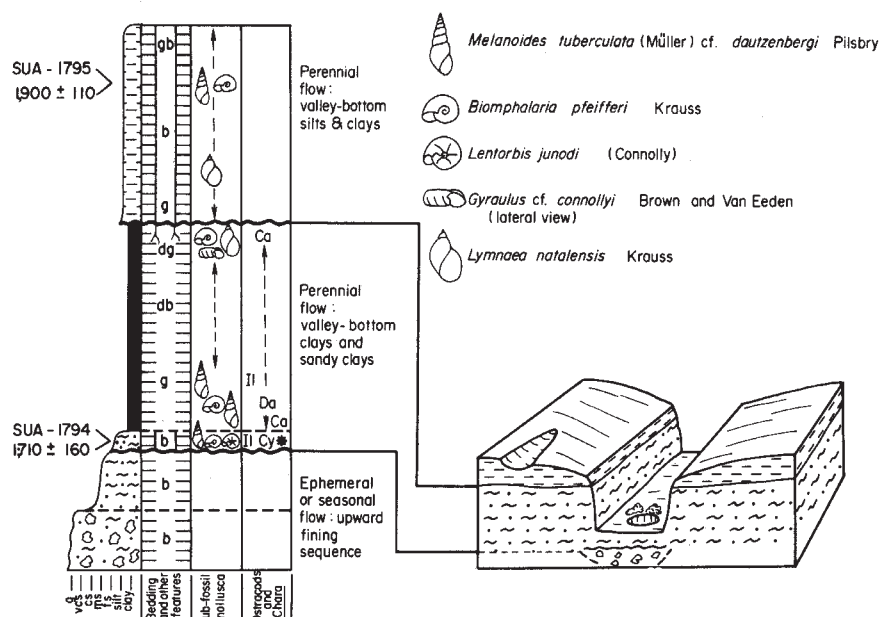


Fig. 2 Stratigraphy and local geomorphic context of the Erkowit shell-bearing clays. The ostracods are *Ilyocypris* sp. (Il), *Cyprinotus* sp. (Cy), *Canodopsis* sp. (Ca) and *Darwinula* sp. (Da). Broken arrows denote scattered distribution. The star denotes *Chara* sp. A and B. Bedding is massive (open dashes) or flat (closed dashes). Colour symbols denote brown (b), grey (g) and dark (d). The lithological symbols are clay as black, silt as dashes, sand as dots, and clay-sand mixtures as dots and wavy lines.

Table 1 Erkowit sub-fossil mollusc collections

- a June 1941 Tothill collection (ref. 1)
Xerophila sp.
Cerastus abyssinica Pfv.
Planorbis herbi Byt.
Bulinus truncatus Aud.
Limnaea caillaudi Boury.
Melanoides tuberculata Mull.
- b January 1973 Williams collection (this paper)
Melanoides tuberculata (Muller) cf. *dautzenbergi* Pilsbry
Lentorbis junodi (Connolly)
Biomphalaria pfeifferi Krauss
Lymnaea natalensis Krauss
Gyraulus cf. *connollyi* Brown & Van Eeden

Note that our *L. natalensis* is probably Tothill's *L. caillaudi*.

evidence point to a regionally wetter climate at about this time.

Short-lived lake transgressions are apparent at Lake Bosumtwi in Ghana soon after 2,400 BP²⁹, at Lake Abhe and other lakes in the Afar desert^{30,31} towards 2,000–1,500 BP, and in the Ziway–Shala basin³² of the Ethiopian Rift at ~1,600 BP. In the Chad basin several small interdune lakes are dated at 2,400 BP and 1,750 BP, at which time the Bahr el Ghazal had started to flow again^{33,34}. There are also signs of reduced aridity in southern Libya between 1,930 and 1,435 BP, coeval with runoff from Tibesti³⁵ and with alluviation in the Hoggar³⁶.

In the Sudan, swamps reached to the foot of Jebel el Tomat on the lower White Nile during 1,930–1,600 BP³⁷. The twin hills are now over 12 km from the closest riparian swamps³⁸. The two centurions sent by Nero about AD 60 to find the sources of the White Nile reported that their way was blocked by vast swamps—the present Sudd. If the two rock outcrops that they saw within the swamp are indeed Jebel Ahmed Agha, as seems plausible, these are now 200 km beyond the northern edge of the Sudd³⁹. The extensive iron smelting at Meroe (Fig. 1) presupposes abundant fuel for charcoal⁴⁰, and out in the Butana present runoff is not enough to fill the large Meroitic earth dams³⁹, nor to sustain much settlement^{40–42}. Diodorus Siculus³⁹ wrote soon after 60 BC of cave-dwelling cattle herders in the Red Sea Hills who fought each other for pasture and retreated into swampy places. There are no such swamps today. In Alexandria at about this time Ptolemy recorded rare summer rains from southerly winds³⁹, suggesting that the Inter-Tropical Convergence Zone on occasion reached further north than it does today.

Taken together, the limnological, archaeological and archival evidence from Sudan, Egypt, Ethiopia, Chad, Libya and Ghana is entirely consistent with a regional climate somewhat wetter than today at about the time that freshwater molluscs were able to live in the now arid Red Sea Hills of eastern Sudan some 2,000 years ago.

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- Butzer, K. W., Isaac, G. L., Richardson, J. L. & Washbourn-Kamau, C. *Science* **175**, 1069–1076 (1972).
- Street, F. A. & Grove, A. T. *Quat. Res.* **12**, 83–118 (1979).
- Singh, G., Joshi, R. D., Chopra, S. K. & Singh, A. B. *Phil. Trans. R. Soc.* **B267**, 467–501 (1974).
- Kershaw, A. P. *New Phytol.* **69**, 785–805 (1970).
- Williams, M. A. J. *Nature* **253**, 617–618 (1975).
- Sarnthein, N. *Nature* **272**, 43–46 (1978).
- Smith, A. B. in *The Sahara and the Nile* (eds Williams, M. A. J. & Faure, H.) 489–502 (Balkema, Rotterdam, 1980).
- Clark, J. D. in *The Sahara and the Nile* (eds Williams, M. A. J. & Faure, H.) 527–582 (Balkema, Rotterdam, 1980).
- Suzuki, H. *Bull. Dept Geogr. Univ. Tokyo* **9**, 29–63 (1982).
- McIntosh, S. K. & McIntosh, R. J. *Am. Scient.* **69**, 602–613 (1981).
- Tothill, J. D. *Sudan Not. Rec.* **27**, 153–183 (1946).
- Andrews, W. F. in *Agriculture in the Sudan* (ed. Tothill, J. D.) 32–61 (Oxford University Press).
- Barbour, K. M. *The Republic of the Sudan*, 45, 72, 226 (University of London Press, 1961).

- Sudan Meteorological Service. *Climatological Normals, 1921–1950* (Khartoum, 1957).
- Griffiths, J. F. in *Climates of Africa* (ed. Griffiths, J. F.) 133–165 (Elsevier, London, 1972).
- Van Markhoven, F. P. C. M. *Post-Palaeozoic Ostracodes—Their Morphology, Taxonomy and Economic Use Vol. II* (Elsevier, London, 1963).
- Peck, R. E. *Bot. Rev.* **19**, 209–227 (1953).
- Wray, J. L. in *Calcareous Algae: Developments in Palaeontology and Stratigraphy Vol. 4*, 110 (1977).
- Sandford, K. S. *Q. Jl. geol. Soc. Lond.* **92**, 201–220 (1935).
- Gardner, E. W. *Mém. Inst. Egypte* **18**, 1–123 (1932).
- Brown, D. S. *Bull. Br. Mus. nat. Hist. (Zool.)* **12**, 39–94 (1965).
- Wright, C. A. *Bull. Br. Mus. nat. Hist. (Zool.)* **10**, 259–274 (1963).
- Wright, C. A. *Bull. Br. Mus. nat. Hist. (Zool.)* **10**, 449–528 (1963).
- Brown, D. S. *Freshwater Snails of Africa and their Medical Importance*, 1–487 (Taylor & Francis, London, 1980).
- Ayad, N. *Bull. Wld Hlth Org.* **14**, 1–117 (1956).
- Sturrock, R. F. *Annls trop. Med. Parasit.* **60**, 100–105 (1966).
- Williams, S. N. & Hunter, P. J. *Bull. Wld Hlth Org.* **39**, 949–954 (1968).
- Brown, D. S. & Lemma, A. *Annls trop. Med. Parasit.* **64**, 533–538 (1970).
- Talbot, M. R., Livingstone, D. A., Palmer, P. G., Maley, J., Melack, J. M., Delibrias, G. & Gulliksen, S. *Palaeoecol. Afr.* **16** (in the press).
- Gasse, F. *Nature* **265**, 42–45 (1977).
- Gasse, F. & Street, F. A. *Palaeogeogr. Palaeoclim. Palaeoecol.* **24**, 279–325 (1978).
- Street, F. A. in *Proc. 8th Panaf. Congr. prehist. Quat. Studies Nairobi 1977* (eds Leakey, R. E. & Ogot, B. A.) 143–146 (International Louis Leakey Memorial Institute of African Prehistory, Nairobi, 1980).
- Maley, J. *Études palynologiques dans le bassin du Tchad et paléoclimatologie de l'Afrique nord-tropicale de 30 000 ans à l'époque actuelle 1–586* (Trav. et Doc. No. 129, ORSTOM, Paris, 1981).
- Servant, M. *Séquences continentales et variations climatiques: évolution du bassin du Tchad au Cénozoïque supérieur*, 1–573 (Trav. et Doc. No. 159, ORSTOM, Paris, 1983).
- Pachur, H.-J. & Braun, G. *Palaeoecol. Afr.* **12**, 351–363 (1980).
- Rognon, P. *L'Atakor et ses bordures: étude géomorphologique*, 1–559 (CNRS, Paris, 1967).
- Clark, J. D. & Stemler, A. *Nature* **254**, 588–591 (1975).
- Adamson, D. A., Gillespie, R. & Williams, M. A. J. in *A Land Between Two Niles* (eds Williams, M. A. J. & Adamson, D. A.) 165–219 (Balkema, Rotterdam, 1982).
- Jackson, J. K. *Sudan Not. Rec.* **38**, 47–66 (1957).
- Shinnie, P. L. *Meroe: A Civilisation of the Sudan*, 1–229 (Thames & Hudson, London, 1967).
- Ali, Ahmed M. in *Man, Settlement and Urbanism* (eds Ucko, P. J., Tringham, R. & Dimbleby, G. W.) 639–646 (Duckworth, London, 1972).
- Marks, A. E., Ali, Abbas M., Hays, T. R. & Elamin, Yousif *Nyame Akuma* **22**, 26–27 (1983).

Nutrient depletion indicates high primary productivity in the Weddell Sea

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The Southern Ocean, and in particular the Weddell Sea, have long been considered areas of high biological productivity¹, but recent isotopic measurements of primary productivity have not confirmed this view^{2,3}. Because the large zooplankton and marine mammal populations of the Southern Ocean depend ultimately on phytoplankton as the base of the food web, accurate knowledge of primary productivity is essential to our understanding of the Antarctic ecosystem. Oceanographic data collected aboard the Soviet icebreaker *Mikhail Somov* have allowed us to derive a new productivity estimate, based on the seasonal depletion of nitrate, phosphate and silicic acid in the surface layer. From these depletions and data on the elemental composition of Southern Ocean phytoplankton, we estimate average primary productivity in the Weddell Sea in the spring-time to be 220–420 mg C m⁻² day⁻¹. Our most conservative estimate is 1.5–4 times higher than recently reported measurements of productivity in the open ocean areas of the Southern Ocean^{2–5}. Our estimates are inherently averages over time and space, including the effects of brief, intense spring blooms of phytoplankton which may occur near the receding ice edge^{6–8}. Studies of primary productivity based on isotope uptake experiments, particularly in the austral summer, may fail to account for the significance of such blooms.

In October and November 1981, the joint US–Soviet Weddell Polynya Expedition aboard *Mikhail Somov* penetrated several hundred kilometres into the seasonal pack ice covering the eastern Weddell Sea, roughly along the Greenwich meridian. Preliminary results, including a description of the hydrographic regime and sea ice conditions, have been reported elsewhere^{9–12}.

The sub-ice surface waters comprised a very homogeneous mixed layer some 60–110 m thick in which dissolved nutrient concentrations were highest at the northernmost stations, decreasing slightly in the Weddell gyre proper. Because ice conditions restricted *Somov* to a relatively small sector of the northeastern Weddell gyre, it was necessary to examine the data set in the context of data from other cruises in the same area. Comparison of the nutrient data with austral summer data from GEOSECS and *Islas Orcadas* revealed that the austral summer data exhibited evidence of marked surface layer nutrient depletions. It was also apparent that virtually unaltered remnants of winter surface waters were present at several of the austral summer stations¹². The existence of this unaltered winter water¹³ (WW) at GEOSECS station 89, near the *Somov* track, with nutrient concentrations very similar to those in the winter surface layer, led us to derive an estimate of phytoplankton productivity from the net depletion of nutrients in the overlying surface waters.

Several assumptions are implicit in this approach to the estimation of seasonal nutrient depletion: (1) the separate summer and winter data sets (that is, different cruises) are quantitatively compatible; (2) little or no vertical or lateral mixing takes place to alter nutrient concentrations in either the surface layer or the WW layer beneath it; (3) a realistic time frame can be assigned over which the depletion of nutrients occurs; (4) vertical homogeneity in the surface layer under the ice is characteristic of late winter conditions in all years.

The hydrographic data used here were collected on the GEOSECS Atlantic cruise in 1972–73, *Islas Orcadas* cruise 12 in 1977 and the *Somov* cruise in 1981. The nutrient data from the GEOSECS and *Somov* cruises were obtained using the same automated techniques¹⁴; on *Islas Orcadas* manual techniques were used¹⁵. Intercomparisons of the nutrient chemical data on deep potential temperature and density surfaces confirm the comparability of the GEOSECS and *Somov* data, but the *Islas Orcadas* data appear to be systematically lower in dissolved silicic acid and higher in phosphate. In our calculations, we have used the *Islas Orcadas* data solely to illustrate the spatial extent of the nutrient depletion. Table 1 presents the temperature, salinity and dissolved nutrient data (phosphate, nitrate and silicic acid) we used.

Table 1 and Fig. 1 illustrate the evidence for stating that minimal vertical diffusion has occurred to alter the dissolved nutrient concentrations in either the surface layer or the WW. The *Somov* data show the temperature of the entire surface mixed layer beneath the ice to be almost at the freezing point. It is underlain by warmer, more saline water and would be heated from above by insolation as the ice recedes; thus, any substantial vertical mixing across the WW layer would cause an increase in its temperature. No such increase was observed (Fig. 1). Instead, a cold lens of remnant WW exists in the Weddell

Sea. This was first noted half a century ago¹⁶ but very few chemical observations have been available until recently. For the depletion calculations discussed here, we have used only the GEOSECS station at which the WW properties were demonstrably identical to those in the wintertime mixed layer (Fig. 1).

Generally, horizontal advection and mixing could also modify nutrient concentrations in surface waters above the remnant WW layer, but in the Weddell gyre lateral transport would do little to alter the apparent depletion of the surface waters relative to the underlying WW. There is a high degree of lateral homogeneity in the Weddell Sea, particularly in the zonal direction in which the major circulation occurs¹⁶. Additionally, circulation within the gyre is quite weak^{9,17}, and vertical shear between the surface and WW is not sufficient for the surface waters observed in the austral summer to have originated very far away from the underlying WW.

Dilution of the surface layer by melt water from the receding ice pack (mean thickness = 1 m) would cause an apparent depletion of nutrients in the surface layer. Adding 1 m of melt water to a surface layer 40 m thick (that is, a dilution of 2.5%) would account for ~7% of the observed nutrient depletion. Some of the *Islas Orcadas* stations we examined had surface salinities which were diluted by as much as 5% relative to the WW, which could account for 15–20% of the apparent nutrient depletion. At the austral summer GEOSECS station used in our calculations, the surface salinity is within 1% of the salinity of the WW layer, indicating that dilution effects were negligible.

The assignment of a time frame during which the observed nutrient depletion took place is somewhat problematical. The timing of the retreat of the pack ice is highly variable from year to year, usually beginning in mid-November^{18,19}. November 1972 had the greatest extent of pack ice for the decade 1966–76¹⁹, and it is thus unlikely that the GEOSECS station location was ice free until late November or early December. (The November ice extent data are the best indication of maximum annual ice extent available for the pre-microwave satellite era¹⁹.) The GEOSECS observations were made during January 1973; therefore, we have assigned a 60–90 day time frame to the observed nutrient depletion.

The annual cooling of the surface layer at the onset of the austral winter results in the formation of a homogeneous surface layer before ice formation¹⁶. The *Somov* data indicate that this layer remains homogeneous in all properties throughout the winter; therefore little or no primary production occurs below the ice to alter the nutrient concentrations. Observations of very low levels of chlorophyll *a* and extremely limited light penetration of the pack ice during the *Somov* cruise²⁰ support the conclusion that primary productivity under the ice pack is minimal. Thus, the homogeneity of nutrient concentrations in the surface mixed layer at the end of austral winter can be assumed to be typical.

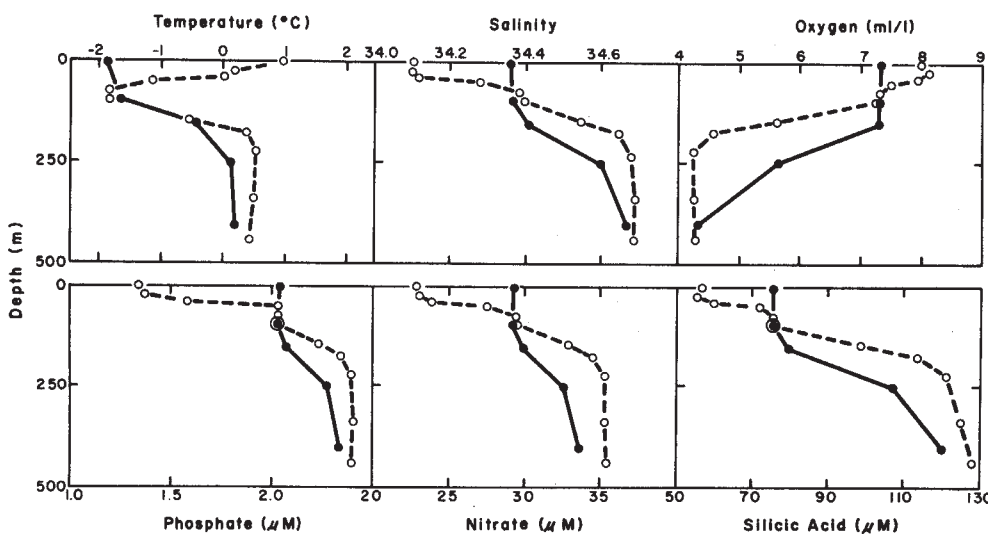


Fig. 1 Hydrographic properties at *Somov* station 35 at 59° 30' S 0° 30' E (solid lines) and GEOSECS station 89 at 60° 15' S 0° 15' E (dashed lines). The remnant winter water at the GEOSECS station is at 75–100 m.

Table 1 Winter water at GEOSECS 89 and wintertime surface water at WEPOLX 35

Station	Pressure (db)	Potential temperature (°C)	Salinity (‰)	Silicate (μM)	Phosphate (μM)	Nitrate (μM)
GEOSECS 89	1	0.965	34.103	56.9	1.34	22.9
22 January 73	75	-1.830	34.383	75.7	2.03	29.4
60° 01' S/0° 01' E						
WEPOLX 35	Entire surface mixed layer	-1.858	34.358	75.7	2.04	29.3
12 November 81						
59° 05' S/0° 40' E						

Figure 2 illustrates the spatial extent of the summer/winter contrast in dissolved silicic acid and phosphate concentrations in the eastern Weddell Sea. Because we believe the *Islas Orcadas* nutrient data to be systematically offset from the *Somov* data, we have used only the GEOSECS data to quantify the observed nutrient depletion. Taking the seasonal depletion to be the difference between the observed nutrient concentration and the WW nutrient concentration, the vertically integrated depletions at GEOSECS station 89 are 850 mmol m⁻², 27 mmol m⁻² and 300 mmol m⁻² for silicic acid, phosphate and nitrate, respectively. The average rates of depletion over the 60 and 90 day time frames assigned are shown in Table 2.

Our average nutrient depletion rates can be compared with experimentally determined rates of nitrogen and silicon assimilation by Antarctic phytoplankton. On recent cruises in the Scotia

Sea^{4,7}, vertically integrated nitrate uptake rates were 0.74–2.41 mmol m⁻² day⁻¹ for those stations which were not in close proximity to a land mass. (An 18-h day has been used to convert from reported hourly rates.) Our most conservative estimate of the average daily nitrate depletion (Table 2) is 2.5–4 times the mean rate for these Scotia Sea stations. Similarly, for stations south of 60° S in the Pacific sector of the Southern Ocean, net production of biogenic silica in the euphotic zone was 2.5–4.5 mmol Si m⁻² day⁻¹ (ref. 21), compared with our calculated silicic acid depletion rates of 9.4–14.2 mmol m⁻² day⁻¹. Therefore, the seasonal average depletion rates of both nitrate and silicic acid exceed by a factor of 1.5–5 the few available directly measured rates in the Southern Ocean.

The estimation of primary productivity from nutrient depletion requires knowledge of the stoichiometry of nutrient assimilation and carbon fixation by phytoplankton. The average composition of marine phytoplankton is often taken to be C/N/P=106/16/1 (ref. 22), but lower C/N and C/P ratios have been reported for Southern Ocean phytoplankton²³. Similarly, C/Si ratios for diatoms in the Southern Ocean are apparently lower than those for most marine diatoms²³. We have used C/N/P=62/11/1 and C/Si=2.5 as representative of Antarctic phytoplankton²³. Our estimates of average primary productivity are shown in Table 2. They range from ~220 mg C m⁻² day⁻¹ to 420 mg C m⁻² day⁻¹.

Our lowest estimates of average primary productivity are based on phosphate and nitrate depletion occurring over a 90-day period. Because particulate nitrogen and phosphorus are remineralized more rapidly than is biogenic silica, they are more likely to be recycled within the euphotic zone. One aspect of this recycling is that some nitrogen is returned to the euphotic zone as ammonium by zooplankton excretion and microbial regeneration (see, for example, ref. 4). This ammonium is utilized preferentially by phytoplankton⁴, so our nitrate-based estimate of primary productivity is inherently an underestimate. The estimate of primary productivity from nitrate depletion is considered to be that fraction of the total which is available for net transport up the food chain^{4,7}. Available data on ammonium and nitrate uptake by Southern Ocean phytoplankton suggest that ammonium utilization supports 40–70% of the total productivity^{4,7}, so our estimate of nitrate utilization probably accounts for no more than half of the total. As only direct measurements of biogenic silica dissolution made in the Southern Ocean indicate that about one-third of the assimilated silicic acid dissolves in the upper 100 m²¹, our silicic acid-based calculation may well be low by that amount. If our nitrate- and silicic acid-based

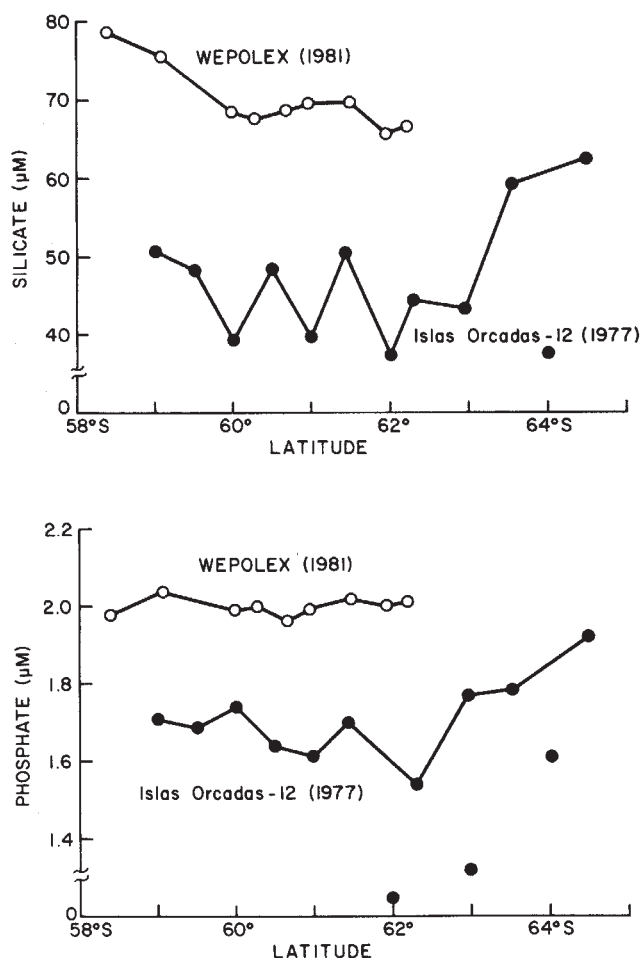


Fig. 2 Meridional distribution of dissolved silicic acid and phosphate in surface waters of the eastern Weddell Sea. *Islas Orcadas* data from summer 1977 are contrasted with *Somov* data from winter 1981 to illustrate the north-south extent of the apparent seasonal depletion.

Table 2 Primary productivity estimated from nutrient depletion

Nutrient	Observed depletion (mmol m ⁻²)	Depletion rate (mmol m ⁻² day ⁻¹)		Estimated primary production (mg C m ⁻² day ⁻¹)
		90-day interval	60-day interval	
Silicic acid	850	9.5	14.2	282–426
Phosphate	27	0.30	0.45	223–335
Nitrate	300	3.4	5.1	223–335

primary productivity estimates are increased accordingly, they agree within 10%.

We believe that our calculations are conservative ones, resulting in estimates of primary productivity that are probably low, yet are 1.5–5 times higher than recent isotopic productivity measurements in the Southern Ocean^{2,3,5,24}. Intense phytoplankton blooms have been reported near the edge of the receding seasonal ice pack which could easily account for much of the discrepancy between our calculated rates and those measured isotopically. Primary productivity in these blooms often exceeded $1 \text{ g C m}^{-2} \text{ day}^{-1}$ (refs 6–8, 24). As its seasonal ice cover recedes, most of the Weddell Sea surface is an ice edge zone for some period of the austral spring. A brief (10–15 day) bloom during which productivity was in excess of 1 g C

$\text{m}^{-2} \text{ day}^{-1}$, followed by a longer period of much lower productivity (closer to the $\sim 0.1 \text{ g C m}^{-2} \text{ day}^{-1}$ values of the recent literature^{2,3,5,24}), would result in seasonal average rates similar to those we derive. The contribution of such blooms is inherently included in our calculations, but may be missed by discrete measurements which are remote from the ice edge.

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- Hart, T. J. *Discovery Rep.* **8**, 3–268 (1934).
- Holm-Hansen, O., El-Sayed, S. Z., Franceschini, G. A. & Cuchel, R. L. in *Proc. 3rd SCAR Symp. Antarctic Biology*, 11–50 (Smithsonian Institution, Washington DC, 1977).
- El-Sayed, S. Z. & Turner, J. T. in *Polar Oceans* (ed. Dunbar, M. J.) 463–503 (Arctic Institute of North America, Calgary, Alberta, 1977).
- Glibert, P. M., Biggs, D. C. & McCarthy, J. J. *Deep-Sea Res.* **29**, 837–850 (1982).
- Slawyk, G. *Aust. J. mar. Freshwat. Res.* **30**, 431–448 (1977).
- El-Sayed, S. Z. in *Biology of the Antarctic Seas Vol. 4* (eds Land, G. & Wallen, I.) 301–312 (American Geophysical Union, Washington DC, 1971).
- Olson, R. J. *Limnol. Oceanogr.* **25**, 1064–1074 (1980).
- Smith, W. O. & Nelson, D. M. in *Proc. 4th Symp. Antarctic Biology* (Elsevier, New York, in the press).
- Gordon, A. L. & Huber, B. A. *J. geophys. Res.* **89** (C1), 641–648 (1984).
- Gordon, A. L., Chen, C. T. A. & Metcalf, W. G. *J. geophys. Res.* **89** (C1), 637–640 (1984).
- Clarke, D. B. & Askey, S. F. *J. geophys. Res.* (in the press).
- Jennings, J. C. Jr, Nelson, D. M. & Gordon, L. I. *Antarct. J. U.S.* **8**, 101 (1982).

- Carmack, E. C. & Foster, T. D. *Deep-Sea Res.* **22**, 711–724 (1975).
- Bainbridge, A. E. *Geosecs Atlantic Expedition Vol. 1* (National Science Foundation, Washington DC, 1981).
- Huber, B. A., Rennie, S. E., Georgi, D. T., Jacobs, S. S. & Gordon, A. L. *Islas Orcadas Reports, Cruise 12, Jan–Feb 1977*, Tech. Rep. CU-2-81-TR2 (Lamont-Doherty Geological Observatory, Columbia University, Palisades, 1981).
- Mosby, H. *Scientific Results of the Norwegian Antarctic Expeditions, 1927–1928*, Vol. 1 (11) (Det Norske Videnskaps-Akademi I, Oslo, 1934).
- Gordon, A. L., Martinson, D. G. & Taylor, H. W. *Deep-Sea Res.* **28A**, 151–163 (1981).
- Gordon, A. L. *J. geophys. Res.* **86**, 493–497 (1981).
- Ackley, S. F. *Int. Ass. hydrol. Sci.* **131**, 129–159 (1981).
- Marra, J. & Boardman, D. C. *Mar. Ecol.-Prog. Ser.* (submitted).
- Nelson, D. M. & Gordon, L. I. *Geochim. cosmochim. Acta* **46**, 491–501 (1982).
- Redfield, A. C., Ketchum, B. H. & Richards, F. A. in *The Sea, Ideas and Observations* Vol. 2, 26–77 (Interscience, New York, 1963).
- Copin-Montegut, C. & Copin-Montegut, G. *Deep-Sea Res.* **25**, 911–931 (1978).
- El-Sayed, S. Z. & Taguchi, S. *Deep-Sea Res.* **28A**, 1017–1032 (1981).

Bacterial chemolithotrophy in the ocean is associated with sinking particles

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The oceanic carbon cycle has traditionally been viewed as a reversible, one step reduction–oxidation reaction ($\text{CO}_2 \rightleftharpoons \text{CH}_2\text{O}$). Principle pathways were thought to involve eukaryotic photoautotrophy and oxygen-dependent bacterial respiration, respectively. However, prokaryotic (cyanobacterial) photoautotrophy is now well documented and has even been proposed as a major carbon pathway^{1–6}. In a previous study of the mesopelagic zone in the North Pacific Ocean⁷, the observed downward fluxes of organic carbon, nitrogen, ATP and RNA suggested production *in situ* of new particulate organic carbon at 700–900 m. Here we present evidence that this is indeed the case and that it is mediated by bacterial chemolithotrophy. Energy for this process may be in part provided by detrital NH_4^+ derived from the downward flux of large particles.

In order to reconcile the mesopelagic zone carbon budget, chemolithotrophic production of organic matter was proposed as a possible mechanism⁷. During the Vertex III sediment trap experiment, we had an opportunity to examine the possibility of bacterial chemolithotrophic production at a station, located in the eastern tropical North Pacific Ocean approximately 330 km off Manzanillo, Mexico ($15^\circ 55' \text{ N}$; $107^\circ 12' \text{ W}$). The Vertex III free floating sediment trap array, used for 20.6 days, consisted of 168 individual collectors at 14 depths (50–2,000 m). Details of the trap construction are given elsewhere⁸. To test the chemolithotrophy hypothesis, we used five sediment trap collectors at each of five depths (100–750 m). Measurements included: (1) concentration of ATP extracted *in situ*⁹ to estimate the

biomass of living microorganisms associated with sinking particles, (2) shipboard-extracted ATP concentrations⁷ in unpreserved traps to estimate the net *in situ* changes in biomass during the experiment (see Table 2), (3) concentrations of NH_4^+ , NO_2^- (see Table 1), (4) organic carbon and nitrogen concentrations⁸, (5) rates of microbial DNA synthesis *in situ*⁷, (6) microbial incorporation of $^{14}\text{CO}_2$ (see Table 2), and (7) microscopic examination of glutaraldehyde-fixed sediment trap samples. The *in situ* ^{14}C -incubation experiments and ATP measurements were performed in paired sediment traps, with and without the addition of a mixture of potential chemolithotrophic substrates (see Table 2).

Several results suggest the activity of chemolithotrophic bacteria at these depths. First, the concentration of NH_4^+ measured in the sediment trap solutions (and hence, the corresponding downward fluxes of particle-associated NH_4^+) increased between 100 and 150 m (Table 1), indicating production of NH_4^+ in association with descending particles, scavenging of NH_4^+ from the water column during descent or horizontal advection of particle-associated NH_4^+ . At greater depths both concentration and flux of NH_4^+ consistently decreased. The estimated NH_4^+ fluxes are considered to be minimum values as no attempt was made to remove associated or covalently bound NH_4^+ from the sinking particles. The flux of NH_4^+ thus calculated was 383 and $149 \mu\text{mol m}^{-2} \text{ day}^{-1}$ at 150 and 550 m, respectively, indicating a loss of $234 \mu\text{mol NH}_4^+ \text{ m}^{-2} \text{ day}^{-1}$ over this depth interval (Table 1). Since NH_4^+ was undetectable ($< 0.05 \mu\text{mol l}^{-1}$) in this portion of the water column, we suggest that the NH_4^+ is consumed either in the water column or within the sinking particles at a rate which is equivalent to the measured rates of change (mean rate of $0.59 \mu\text{mol m}^{-3} \text{ day}^{-1}$ between 150–550 m). The consumption of NH_4^+ (that is, $-\Delta\text{NH}_4^+/\Delta Z$) is associated with the appearance of NO_2^- (Table 1). These data suggest the presence of active bacterial nitrification.

Nitrifying bacteria of the genera *Nitrosomonas* and *Nitrosococcus* were counted using specific immunofluorescence techniques^{10,11}. Nitrifiers were detected in high concentrations in all sediment trap collections and the majority were *Nitrosomonas*-type cells. Nitrifiers were frequently observed in clusters of up to 30 cells, but single and paired cells were also common. The mean cell size varied considerably, but large cells, up to $3 \mu\text{m}$ in diameter, were common.

Table 1 Nutrient concentrations, fluxes and average rates of change over the specified depth intervals

Sediment trap depth (m)	Nutrient concentrations ($\mu\text{mol per sediment trap}$)*		Total flux ($\mu\text{mol m}^{-2} \text{ day}^{-1}$) and average rates of change ($\mu\text{mol m}^{-3} \text{ day}^{-1}$) for each depth interval†					
	NH_4^+	NO_2^-	Organic C flux	($\Delta\text{C}/\Delta\text{Z}$)	Organic N flux	($\Delta\text{N}/\Delta\text{Z}$)	NH_4^+ flux	($\Delta\text{NH}_4^+/\Delta\text{Z}$)
100	13.8	<0.005	3,900	(-34)	609	(-7.1)	172	(+4.2)
150	30.8	<0.005	2,192	(-3.7)	254	(-0.42)	383	(-0.59)
550	12.0	2.3	717	(+0.55)	87	(+0.04)	149	(-1.1)
650	3.4	1.4	772	(+1.5)	91	(+0.32)	42	(-0.28)
750	1.1	1.1	925		123		14	

* NH_4^+ and NO_2^- concentrations were measured in 0.22 μm filtrates of the unpreserved sediment trap solutions receiving no chemolithotrophic additions (see Table 2). Samples were analysed by S. Moore within 4 h after sediment trap recovery using a shipboard Technicon Autoanalyzer system.

† Organic C and N were measured in formalin preserved sediment traps as described by Knauer *et al.*⁸.

The downward flux of particle-associated nitrifiers was consistently high (1.15 to 3.52×10^8 cells $\text{m}^{-2} \text{ day}^{-1}$) throughout the depth range of our experiment (100–750 m). Sediment traps pre-charged with chemolithotrophic substrates and incubated *in situ* produced higher cell densities, with values (relative to the control) ranging from 118% (150 m) to 263% (550 m). If we assume a maximum nitrification rate of $1.4\text{--}2 \times 10^{-12}$ mol NH_4^+ per cell per day (refs 12, 13) the particles collected at 550 m are potentially capable of oxidizing between 160–230 $\mu\text{mol NH}_4^+ \text{ m}^{-2} \text{ day}^{-1}$, a value which agrees with the observed loss of NH_4^+ at 150–550 m (Table 1).

The comparison of *in situ* versus shipboard extracted ATP can be used to ascertain the extent to which net microbial growth had occurred in the unpreserved sediment materials during the experiment⁷. It is evident (Table 2) that the two surface traps (100 and 150 m) exhibited post-incubation decreases in total ATP (that is, shipboard ATP < *in situ* ATP) indicating net microbial death. However, for that region of the water column where NH_4^+ was consumed and NO_2^- was produced (550–750 m), there was an increase in total microbial ATP during the incubation period and hence, net carbon production. The greatest increase occurred at 550 m (Table 2). At all three depths where net growth had occurred (550, 650 and 750 m), we observed an increased biomass yield following the addition of potential chemolithotrophic substrates.

The mean total $^{14}\text{CO}_2$ incorporation (Table 2) also displayed a mid-depth (550 m) maximum and, at all depths but 750 m, the incorporation rates were stimulated by the addition of inorganic substrates. However, $^{14}\text{CO}_2$ incorporation data, by themselves, cannot be extrapolated directly to microbial carbon production without information on the proportion of autotrophic

versus heterotrophic processes. Heterotrophic bacteria normally assimilate 2–10% of their total carbon cell quota as CO_2 (refs 14–16), whereas certain chemolithotrophs obtain potentially 100% of their cell carbon requirements from CO_2 (refs 17, 18). By assuming the ^{14}C -incorporation data represent strictly chemolithotrophic growth, the data can be extrapolated and compared with total microbial carbon production derived from estimates of DNA synthesis (Table 3). The DNA synthesis (^3H -adenine) technique does not discriminate between the two major pathways of carbon synthesis and hence provides an estimate of total microbial carbon production. In the surface sediment traps (100 and 150 m), total microbial production greatly exceeds the ^{14}C -derived estimate, indicating that chemoheterotrophic processes predominate (Table 3). This is consistent with the observed loss of organic carbon and production of NH_4^+ associated with the large sinking particles (Table 1). However, between 550 and 750 m chemolithotrophic carbon production was detected with estimates ranging from 7% (650 m) to 90% (550 m) of the total microbial production and may therefore be a significant, if not dominant pathway for carbon and energy flow at certain depths in the mesopelagic zone.

If we assume that nitrification is the dominant pathway we can calculate a minimum amount of energy required to sustain this observed mesopelagic bacterial production. The oxidation of NH_4^+ to NO_2^- proceeds with a ΔG of $-66 \text{ kcal mol}^{-1}$, and with typical cell yields of 0.03–0.06 mol C produced per mol N oxidized¹⁹. Thus at 550 m, the estimated chemolithotrophic microbial production ($425 \mu\text{g C m}^{-2} \text{ day}^{-1}$) would require a flux of $787 \mu\text{mol NH}_4^+ \text{ m}^{-2} \text{ day}^{-1}$, if nitrifiers were exclusively responsible, which is a value approximately three times the

Table 2 Effects of addition of exogenous reduced inorganic substrates *in situ* to sediment trap collected particles

Sediment trap depth (m)	ATP concentration, <i>in situ</i> extraction (ng per sediment trap)	Chemolithotrophic additions	ATP concentration, shipboard extraction (ng per sediment trap)	%ATP change*	$^{14}\text{C-HCO}_3^-$ incorporation into organic matter (nCi per sediment trap)
100	1,760	No	705 $\pm$ 382	-60	11.0 $\pm$ 0.8
		Yes	1,434 $\pm$ 429	-19	16.1 $\pm$ 1.9
150	1,310	No	750 $\pm$ 6	-43	16.4 $\pm$ 0.5
		Yes	556 $\pm$ 37	-57	30.6 $\pm$ 5.3
550	110	No	748 $\pm$ 94	+580	42.7 $\pm$ 3.0
		Yes	4,004 $\pm$ 543	+3,540	48.7 $\pm$ 7.3
650	65	No	286 $\pm$ 106	+340	6.9 $\pm$ 0.2
		Yes	844 $\pm$ 271	+1,198	18.6 $\pm$ 0.7
750	220	No	662 $\pm$ 298	+201	18.3 $\pm$ 0.6
		Yes	1,198 $\pm$ 184	+445	14.5 $\pm$ 0.8

ATP associated with sinking particles was extracted *in situ* by precharging the sediment traps with a high density acid-salt solution (0.5 M H_3PO_4 , 1.5 M NaCl $\rho = 1.05 \text{ g cm}^{-3}$) as described by Fellows *et al.*⁹. For *in situ* incubations paired sediment traps were prepared for each depth by precharging with 2.5 l of an autoclaved high density solution consisting of 0.22 μm filtered seawater containing 33.8 g NaCl , 14.3 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.93 g KCl and 30 $\mu\text{Ci NaH}^{14}\text{CO}_3$ (50 mCi mmol^{-1} ; New England Nuclear) per litre ($\rho = 1.065 \text{ g cm}^{-3}$). At each depth, one of the paired traps received the chemolithotrophic mix consisting of NH_4Cl , $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ and MnCl_2 to yield final concentrations of 400 $\mu\text{M NH}_4^+$, 400 $\mu\text{M S}_2\text{O}_3^{2-}$ and 100 $\mu\text{M Mn}^{2+}$. Shipboard ATP concentration refers to the value measured in the unpreserved sediment traps after recovery so as to distinguish it from the *in situ* extracted ATP. Details of the methods are presented by Karl and Knauer⁷. For ^{14}C incorporation, triplicate subsamples (100 ml each) were concentrated onto Whatman GF/F filters acidified with HCl , evaporated to dryness *in vacuo*, reconstituted with 1 ml deionized H_2O , mixed with 10 ml Aquasol (NEN) and radioassayed.

* Calculated as: [(shipboard ATP - *in situ* ATP) / *in situ* ATP] $\times$ 100%.

Table 3 *In situ* microbial production of organic matter in association with sediment trap collected particles and estimates of the role of bacterial chemolithotrophy

Sediment trap depth (m)	Chemolithotrophic additions	Minimum ^{14}C -derived microbial production* ($\mu\text{g C m}^{-2} \text{ day}^{-1}$)	Total microbial production† ($\mu\text{g C m}^{-2} \text{ day}^{-1}$)	Estimated % chemolithotrophic based production‡
100	No	110 ± 8	15,900 ± 3,200	< 1
	Yes	160 ± 18		
150	No	163 ± 5.4	9,900 ± 750	2
	Yes	305 ± 53		
550	No	425 ± 30	480 ± 83	90
	Yes	485 ± 66		
650	No	69 ± 2.2	1,000 ± 17	7
	Yes	185 ± 6.6		
750	No	182 ± 5.7	870 ± 31	21
	Yes	144 ± 8.4		

See Table 2 for composition of chemolithotrophic mixture.

* Calculation assumes that CO_2 represents 100% of the carbon cell quota (that is obligately chemolithotrophic) and is based upon the measured initial ^{14}C specific radioactivity of $15 \mu\text{Ci mmol}^{-1}$ of total inorganic C. Values are mean ± 1 s.d.

† Total carbon production extrapolated from *in situ* measurements of rates of DNA synthesis using the conversion factors of Karl and Winn²⁰. Values are mean ± 1 s.d.

‡ Calculated from data obtained without chemolithotrophic additions.

minimum *in situ* NH_4^+ consumption rate estimated from the sediment trap data (Table 1). This rate of NH_4^+ oxidation could consume potentially $1.6 \text{ mmol O}_2 \text{ m}^{-2} \text{ day}^{-1}$, if completely oxidized to NO_3^- . Therefore, chemolithotrophy (bacterial reduction of CO_2 and concomitant O_2 consumption) rather than chemoheterotrophic utilization of surface-derived organic matter may be an important mechanism for the consumption of O_2 in the mesopelagic zone of the ocean. Although we have singled out nitrification as an important metabolic process, other pathways such as the oxidation of CH_4 , H_2S , H_2 and other reduced products of anaerobic decomposition may also contribute energy for the observed mesopelagic production.

These results suggest that the oceanic carbon cycle may be more complex than previously described. The flux of energy descending from the euphotic zone may greatly exceed that nominally contained in particulate organic detritus, which has previously been considered to be the principle factor controlling the rates of mesopelagic microbiological activity and O_2 utilization. Although the flux of energy (in the form of reduced products of bacterial decomposition) required to sustain the chemolithotrophic production measured in this study is probably derived from surface primary production and thus cannot exceed it in steady-state conditions, mesopelagic 'primary' production may have a major impact on the trophic organization of localized mid-water food webs and on the production and maintenance of the intermediate depth O_2 minimum.

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1. Waterbury, J. B., Watson, S. W., Buillard, R. R. & Brand, L. E. *Nature* **277**, 293-294 (1979).
2. Johnson, P. W. & Sieburth, J. M. *Limnol. Oceanogr.* **24**, 928-935 (1979).
3. Krempin, D. W. & Sullivan, C. W. *Can. J. Microbiol.* **27**, 1341-1344 (1981).
4. Morris, I. & Glover, H. *Limnol. Oceanogr.* **26**, 957-961 (1981).
5. Li, W. K. *et al. Science* **219**, 292-295 (1983).
6. Platt, T., Subba Rao, D. V. & Irwin, B. *Nature* **301**, 702-704 (1983).
7. Karl, D. M. & Knauer, G. A. *Deep-Sea Res.* (in the press).
8. Knauer, G. A., Martin, J. H. & Bruland, K. W. *Deep-Sea Res.* **26A**, 97-108 (1979).
9. Fellows, D. A., Karl, D. M. & Knauer, G. A. *Deep-Sea Res.* **28A**, 921-936 (1981).
10. Ward, B. B. & Perry, M. J. *Appl. envir. Microbiol.* **39**, 913-918 (1980).
11. Ward, B. B. *J. mar. Res.* **40**, 1155-1172 (1982).
12. Watson, S. W. *Limnol. Oceanogr.* **10**, R274-289 (1965).
13. Carlucci, A. F. & Strickland, J. D. H. *J. exp. mar. Biol. Ecol.* **2**, 156-166 (1968).
14. Kornberg, H. L. *Symp. Soc. gen. Microbiol.* **15**, 8-39 (1965).
15. Romanenko, V. I. *Microbiology* **34**, 334-339 (1965).
16. Overbeck, J. *Arch. Hydrobiol. Beih. Ergebn. Limnol.* **13**, 56-61 (1979).
17. Peck, H. D. A. *Rev. Microbiol.* **22**, 489-518 (1968).
18. Kelly, D. P. A. *Rev. Microbiol.* **25**, 177-210 (1971).
19. Painter, H. A. *Water Res.* **4**, 393-450 (1970).
20. Karl, D. M. & Winn, C. D. in *Heterotrophic Activity in the Sea* (eds Hobbie, J. E. & Williams, P. J. leB.) (Plenum, New York, in the press).

Human large granular lymphocytes are potent producers of interleukin-1

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Natural killer (NK) activity¹ against tumour and virus-infected target cells is shown by a subpopulation of peripheral blood mononuclear leukocytes with the morphological features of large granular lymphocytes (LGL)². The lineage of human LGL is still controversial, as they display surface markers of both T lymphocytes and myelomonocytic cells³. LGL have recently been reported to produce lymphokines such as interleukin-2 (IL-2)^{4,5} and α - as well as γ -interferons⁶, functions associated mainly with T cells. To determine whether cytokines associated with other cell lineages are also produced by LGL, we examined whether they might produce a myelomonocyte-associated cytokine such as interleukin-1 (IL-1). IL-1 is a 12-18,000 molecular weight (MW) lymphokine produced by a variety of cell types such as monocytes⁷, keratinocytes⁸ and a human dendritic cell line⁹, which plays a crucial role in immunoregulation and inflammation^{10,11}. Moreover, IL-1 has recently been reported to act synergistically with IL-2 and interferons in boosting LGL-mediated NK activity¹². We now show that a subset of highly purified human LGL with NK activity can be stimulated to secrete a soluble factor with the biochemical and biological characteristics of human IL-1.

LGL were isolated from healthy volunteers by sequential elutriation, removal of adherent cells on a plastic surface and nylon wool columns, and centrifugation on a discontinuous Percoll density gradient². Cells from the resulting fractions were tested for lysis of NK-susceptible tumour target cells and for

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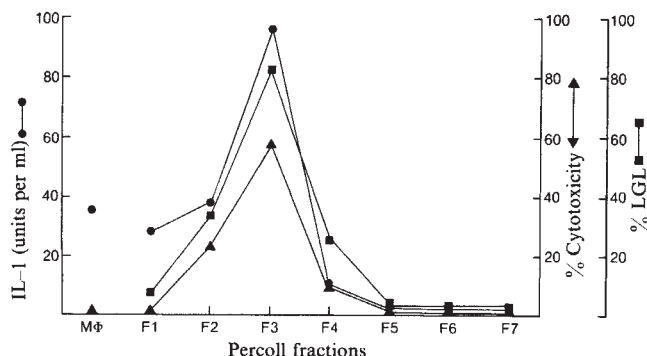


Fig. 1 IL-1 production and NK activity in fractions of peripheral blood lymphocytes separated by discontinuous Percoll density centrifugation. Peripheral blood lymphocytes were isolated and fractionated by centrifugal elutriation and lymphocyte-enriched fractions were depleted of adherent cells first by incubation on autologous serum-coated plastic flasks for 1 h at 37 °C and second, by passage over nylon wool columns. The nonadherent cells were then fractionated on a seven-step discontinuous Percoll (Pharmacia) density gradient². RPMI 1640 medium + 10% fetal calf serum (FCS) and Percoll were adjusted to 285 mosmol per kg H₂O. Seven different concentrations of Percoll in medium were prepared, ranging from 40 to 57% Percoll, each varying from the next by 2.5% concentration steps. 1×10^6 peripheral blood lymphocytes were applied to each gradient and centrifuged at 550g for 30 min at 20 °C. The seven fractions were collected and washed. NK activity was measured in a 4 h ⁵¹Cr-release assay against K562 at an effector:target ratio of 50:1 (ref. 2). For IL-1 production of 1×10^6 ml⁻¹ in RPMI 1640 supplemented with 2 mM glutamine, 2 mg ml⁻¹ HSA (American Red Cross) and 50 µg ml⁻¹ gentamicin. Adherent monocytes were detached from the surface of plastic flask by flushing with cold PBS containing 10% FCS and resuspended at 1×10^6 ml⁻¹ in the same human serum albumin-supplemented medium. Cells were then cultured for 48 h (5% CO₂, 37 °C) in the presence of 25 µg ml⁻¹ LPS. Supernatants were collected, dialysed extensively against PBS (0.15 M NaCl, 0.02 M phosphate, pH 7.2) containing 0.01% polyethylene glycol (PEG) and 50 µg ml⁻¹ gentamicin. The filtered samples (0.22 µm, Millex) were tested for IL-1 activity in a thymocyte co-stimulator assay, as described previously³⁰. A single-cell suspension of C3H/HeJ (*H-2^k*) mouse thymocytes were cultured for 72 h at 1.5×10^6 cells per well in 96-well flat-bottomed plates in the presence of 0.5 µg ml⁻¹ concanavalin A and with serial dilutions of the test samples or a standard preparation of IL-1. Results from triplicate cultures were plotted on linear regression against the standard preparation of IL-1 (set up at 100 units ml⁻¹) and expressed in units ml⁻¹. In every IL-1 assay a sample of medium with 25 µg ml⁻¹ LPS was tested and was consistently negative (< 1 unit ml⁻¹). Each sample from this and the following experiments was negative for IL-2 activity when tested on the IL-2-dependent CT6 cell line³¹ and was found free of contaminating IL-2. This experiment was repeated four times with similar results. The percentage of LGL of each fraction of Percoll gradient was determined by morphological examination of Giemsa-stained cytopreps². MΦ, macrophages.

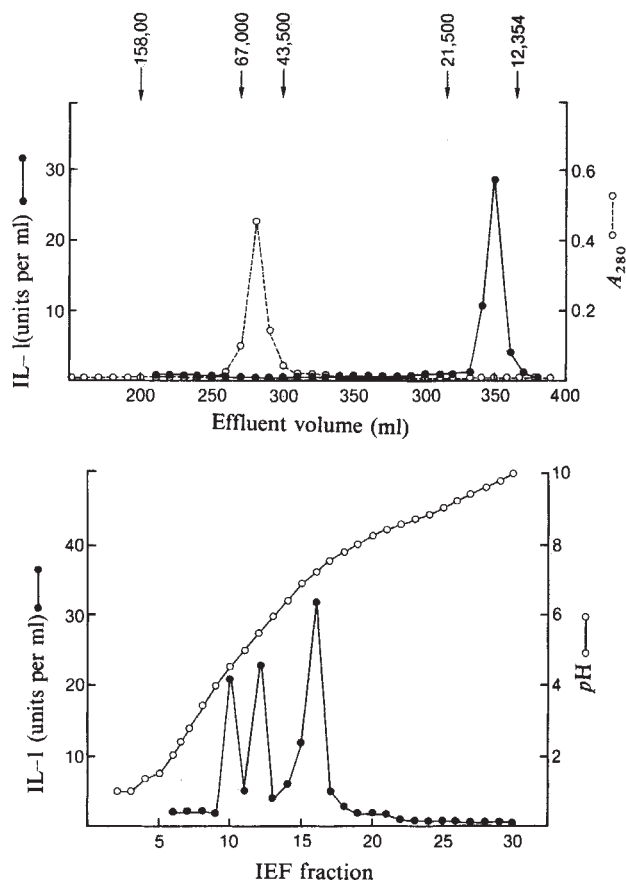


Fig. 2 Biochemical characterization of IL-1 derived from cultured LGL. 6×10^7 LGL, collected after 3 weeks' culture as described in Table 1, were washed three times with RPMI 1640 and cultured for 48 h (5% CO₂, 37 °C) in the presence of 25 µg ml⁻¹ LPS at 2×10^6 cell ml⁻¹. Supernatants were collected and 47.6 g (NH₄)₂SO₄ per 100 ml supernatant were gradually added under gentle stirring to a final concentration of 75%. The precipitated proteins were collected by centrifugation at 5,000g for 10 min, resuspended in PBS and dialysed extensively against PBS (with 0.01% PEG and 50 µg ml⁻¹ gentamicin). The sample was concentrated using an Amicon ultrafiltration unit with PM10 Diaflo membrane (Amicon) and dialysed against PBS as above. Aliquots of 2 ml were applied to a Sephacryl S200 (Pharmacia) packed column (2.5 × 90 cm). The column was calibrated with the following markers: aldolase (158,000 MW), bovine serum albumin (67,000 MW), ovalbumin (43,500 MW), soybean trypsin inhibitor (21,500 MW), and cytochrome c (12,354 MW). The sample was eluted at a flow rate of 20 ml h⁻¹ with PBS (0.01% PEG, 50 µg ml⁻¹ gentamicin). Aliquots (3 ml) were collected, filtered and tested for IL-1 activity as described in Fig. 1 legend. The fractions active in the IL-1 assay (Fig. 2, upper panel) were pooled, concentrated by Amicon ultrafiltration unit as described above, and dialysed extensively against 0.01% PEG. The sample was loaded onto a preparative isoelectric focusing column (LKB) in a 100 ml of 5–50% sucrose gradient with 0.01% PEG and 2% pH 3.5 to 10 ampholites (LKB). The pH gradient was formed over a period of 18 h at 1,600 V. 3-ml fractions were collected and the pH was determined (Fig. 2, lower panel). Fractions were dialysed extensively against PBS (0.01% PEG, 50 µg ml⁻¹ gentamicin), filtered and tested for IL-1 activity as described in Fig. 1 legend.

production of IL-1 upon stimulation with lipopolysaccharide *Escherichia coli* 055 B:55 (LPS) (Fig. 1). Cytochemical and cell surface analysis revealed that fraction 3 contained predominantly LGL (80–85%), enriched in cells with NK-associated markers (67% OKM1⁺, 62% OKT10⁺, 75% B73.1⁺), but depleted of cells with non-NK associated markers (5% OKT3⁺, $< 1\%$ surface Ig⁺) and contained no detectable monocytes ($< 1\%$ esterase-positive cells)^{3,13}. LPS stimulated IL-1 secretion by cells in fractions 1 and 2 which contained monocytes (30–40% and 5–10% respectively, other cells being large agranular lymphocytes) and in fractions 3 and 4 which were enriched in LGL but depleted of monocytes ($< 5\%$) (Fig. 1). On a per cell basis, the LGL-enriched fraction 3 produced threefold higher levels of IL-1 than the monocyte-containing populations and substantially more than a purified population of adherent monocytes. Moreover addition of $> 5\%$ of autologous adherent monocytes to LGL decreased the production of IL-1 from 164 units per ml to 33 units per ml (data not shown).

LGL-enriched populations from fraction 3 were purified further to remove T cells and macrophages by treatment with the monoclonal antibodies anti-OKT3 (T-cell specific¹⁴) and anti-Leu-M1 (monocyte-granulocyte specific¹⁵) + complement (C). After this treatment, LGL ($\leq 1\%$ OKT3⁺, $< 0.3\%$ Leu-M1⁺) showed significantly greater LPS-induced IL-1 activity on a per cell basis than untreated LGL (Table 1).

The data in Table 1 suggested that OKT3⁺ T cells and Leu-M1⁺ macrophages might suppress production of IL-1 by LGL.

Table 1 Identification of the subset(s) of LGL producing IL-1

	Conditions	% Recovery ($\frac{\text{Final cell no.}}{\text{Initial cell no.}} \times 100$)		IL-1 (units ml ⁻¹) LGL (P)	Macrophages	NK activity* (LU) per cell
		LGL	Macrophages			
Expt 1	C	96	95	142 (-)	72	372
	anti-Leu-M1 and anti-OKT3+C	86	7	190 (<0.05)	<1	405
	anti-Leu-M1 and anti-OKT4+C	92	ND	145 (>0.1)	ND	390
	anti-Leu-M1 and anti-OKT8+C	90	ND	309 (<0.01)	ND	239
Expt 2	LGL			102		222
	LGL OKT11 ⁺			70		225
	LGL OKT11 ⁻			103		166
	LGL B73.1 ⁺			135		321
	LGL B73.1 ⁻			15		6
	LGL			125		188
	LGL OKM1 ⁺			168		481
	LGL OKM1 ⁻			14		2
	LGL			156		240
	LGL DR ⁺			198		180
	LGL DR ⁻			2		215
	LGL+C	88		150.7		160
Expt 3	T cells+C	90		<1		20

Expt 1: LGL were isolated by discontinuous Percoll gradient (fraction 3) and washed three times with RPMI 1640. Aliquots were treated with optimal cytotoxic concentrations of the indicated antibody + complement (low Tox-H rabbit complement, Cedarlane) at 1:10 final dilution. Cells were washed 3× with RPMI 1640 and resuspended in human serum albumin (HSA) supplemented medium at 1×10^6 per ml. Adherent monocytes were resuspended with cold phosphate-buffered saline (PBS) containing 10% fetal calf serum (FCS) and treated with the monoclonal antibodies as indicated. Both LGL and macrophages were stimulated with $25 \mu\text{g ml}^{-1}$ LPS as described in the legend to Fig. 1. The significance level of the data, related to controls, was calculated by Duncan's multiple range analysis of variance. *P* values of ≥ 0.1 were considered not significant. Data are expressed as the mean of six separate experiments. **Expt 2:** LGL from fraction 3 of Percoll gradient were treated with optimal cytotoxic concentrations of anti-OKT3 and anti-Leu-M1 antibodies + C. The resulting OKT3⁻ Leu-M1⁻ LGL were treated with optimal concentrations of the antibodies shown ($5\text{--}10 \mu\text{g ml}^{-1}$) and then labelled with F(ab)₂ goat anti-mouse fluoresceinated antibody (Cappel) at 1:10 final concentration. Cells were analysed and sorted in positive and negative fractions by flow cytometry (Ortho Cytofluorograph). When reanalysed, the positive fraction contained more than 98% positive cells. The negative fraction contained less than 2% positive cells. In all the experiments, the negative cell population was further treated with cytotoxic concentrations of the same antibody + C. Cells were stimulated as described in the legend to Fig. 1. This experiment was repeated twice with similar results. **Expt 3:** LGL (fraction 3 of Percoll gradient) and T lymphocytes from Percoll fraction 7 were cultured *in vitro* for 10 generations in medium supplemented with IL-2. Lectin-free human IL-2 (ABS) was used at 10% v/v. After 3 weeks cells were collected. These cultured LGL were treated with anti-Leu-M1 antibody and C as described in Table 1 legend. The cells, free of contaminating LeuM1⁺ or nonspecific esterase-positive cells, were then stimulated for IL-1 production as described in Fig. 1 legend. IL-1 activity was determined as reported in Fig. 1 legend. This experiment was repeated three times with similar results. ND, not determined.

* NK activity was measured against K562 cell line in a standard 4-h ⁵¹Cr-release assay at different effector: target ratios. Lytic units (LU) were calculated at 30% specific lysis per 10^7 effector cells.

Table 2 Enhancing activity of LGL-derived IL-1 on T-lymphocyte response to PHA and on fibroblast proliferation

IL-1 (units per ml)	T-lymphocyte + PHA (mean c.p.m. $\pm$ s.e.m.)	CRL 1445 fibroblast (c.p.m. $\pm$ s.e.m.)
0	760 $\pm$ 88	1,270 $\pm$ 90
10	8,571 $\pm$ 720	3,819 $\pm$ 228
20	14,492 $\pm$ 650	4,959 $\pm$ 520
40	15,924 $\pm$ 790	9,863 $\pm$ 454
80	21,038 $\pm$ 1,120	10,026 $\pm$ 760

OKT3⁻, LeuM1⁻ LGL were stimulated with $25 \mu\text{g ml}^{-1}$ LPS at 2×10^6 cells per ml for 48 h. Supernatants were collected, dialysed extensively and tested for IL-1 activity in C3H/HeJ mouse thymocyte assay as described in the legend to Fig. 1. Monocyte-depleted T lymphocytes from fraction 7 of Percoll density gradient were cultured at 2.5×10^5 cells per well in the presence of PHA $2.5 \mu\text{g ml}^{-1}$ and the indicated concentrations of LGL-derived IL-1 for 3 days. Cultures were pulsed for the last 8 h with $1 \mu\text{Ci } ^3\text{H-thymidine}$ ($1.9 \text{ mCi mmol}^{-1}$). Results are expressed as mean $\pm$ s.e.m. of triplicate cultures. This experiment was repeated twice with similar results. CRL 1445 human dermal fibroblasts (American Type Culture Collection) at third passage were plated in 96-microwell plates at 1×10^4 cells per well in Dulbecco's modified Eagle's medium containing 10% heat-inactivated (56 °C, 30 min) fetal calf serum, 4 mM glutamine, 20 mM HEPES, $50 \mu\text{g ml}^{-1}$ gentamicin. CLL 1465 fibroblasts were then cultured with LGL-derived IL-1 for 3 days²³. Cultures were pulsed for the last 16 h with $1 \mu\text{Ci } ^3\text{H-thymidine}$ ($1.9 \text{ mCi mmol}^{-1}$). After treatment with trypsin-EDTA solution (0.5 mg ml^{-1} trypsin, 0.2 mg ml^{-1} EDTA, 37 °C, 10 min), cells were collected using an automated device. Results are expressed as the mean $\pm$ s.e.m. of triplicate cultures. This experiment was repeated twice with similar results.

To identify the cell subset which was responsible for the observed suppression, we treated LGL with anti-Leu-M1 + C and then either with anti-OKT4 (reactive with a helper T-cell subset¹⁶ and 4–5% of LGL³) or anti-OKT8 (reactive with a suppressor T-cell subset¹⁷ and 15–20% of LGL³) monoclonal antibodies and C. While removal of OKT4-positive cells had no effect (see Table 1), the IL-1 production was more than doubled by treating LGL with anti-OKT8 monoclonal antibody, suggesting that a population of OKT8⁺ LGL and OKT8⁺ lymphocytes may regulate the IL-1 production by LGL.

To identify the subset(s) of LGL producing IL-1, OKT3⁻, Leu-M1⁻, LGL were sorted by fluorescence-activated cell sorter (FACS) according to their reactivity with anti-OKT11 (ref. 3), anti-B73.1 (ref. 13), anti-OKM1 (ref. 18) or AF-10 (ref. 19); a monoclonal antibody specific from DR surface molecules on macrophages and B cells + C. The results (Table 1) showed that the IL-1 production in response to LPS was predominantly attributable to a B73.1⁺, OKM1⁺, DR⁺ subset of LGL, while the B73.1⁻, OKM1⁻, DR⁻ cell subsets produced only a small level of IL-1 activity. The results also showed that both OKT11⁺ and OKT11⁻ subsets of LGL produced comparable levels of IL-1 activity.

Human LGL maintaining NK activity can be cultured for several weeks in lectin-depleted IL-2 containing medium²⁰. LGL-enriched fraction 3 and T lymphocyte-enriched fraction 7 from discontinuous Percoll density gradients were cultured in IL-2-containing media for about 10 generations. These cultured LGL and T cells were stimulated with LPS, and IL-1 production was determined during a 2-day culture period. LGL cultured for up to 3 weeks maintained their NK activity as previously reported²⁰, as well as their capacity to produce consistently

significant levels of IL-1 activity in the absence of any contaminating monocytes (Table 1). In contrast, cultured T cells did not produce any detectable IL-1 activity and showed no significant NK activity.

In the LGL subset(s) reactive with anti-B73.1 or anti-OKM1 monoclonal antibodies, NK activity correlated with the levels of IL-1 production. The relationship of NK activity and IL-1 production of cells separated on the basis of reactivity with anti-OKT11 monoclonal antibody was evaluated. Both the OKT11⁺ and OKT11⁻ subsets of LGL secreted IL-1 and showed NK activity. In the case of AF-10, however, NK activity was nearly equally distributed in both the DR⁻ and DR⁺ subsets of LGL as reported elsewhere³, whereas the IL-1 production was limited to the DR⁺ subset.

The IL-1 activity of supernatants from cultured LGL (~6 × 10⁷) stimulated with LPS for 48 h was characterized biochemically by sequential (NH₄)₂SO₄ precipitation, Sephacryl S200 gel filtration and preparative isoelectrofocusing (IEF). IL-1 activity derived from cultured LGL eluted from S200 gel column in the range of 15,000 molecular weight (MW) and was heterogeneous on IEF with a major peak at a pI 6.8 and two minor peaks at pI 5.5 and 4.5 (Fig. 2). The IL-1 activity in supernatants derived from fresh LGL had almost identical characteristics (data not shown). These data are consistent with those reported for monocyte-derived human IL-1 (ref. 21).

Monocyte-derived human IL-1 has been reported to enhance the T-lymphocyte response to polyclonal stimulants such as phytohaemagglutinin A (PHA) in the apparent absence of macrophage accessory cells²² and to stimulate human fibroblast proliferation²³. Similarly, LGL-derived human IL-1 could replace the requirement of T-lymphocyte response to PHA as well as to stimulate the proliferation of CRL 1445 human fibroblasts (see Table 2).

IL-1 promotes the generation of cytotoxic effector cells²⁴, has a crucial role in T- as well as B-lymphocyte responses to antigens^{25,26}, stimulates fibroblast proliferation²³ and collagenase production²⁷, and induces fever²⁸ and production of acute phase proteins²⁹. The evidence that LGL produce considerable IL-1 activity suggests that some immunological function(s) in addition to NK lytic activity may be exerted by a subset(s) of LGL. Indeed, we have obtained data showing that the IL-1-producing subset(s) of LGL, OKM1⁺, DR⁺, can also exert an effective accessory cell function for T-lymphocyte activation (data not shown).

In conclusion, our results show that LGL with NK activity can produce IL-1. They show further that IL-1 was secreted mainly by LGL bearing the OKM1⁺ or B73.1⁺ phenotypes, which are those highly associated with NK activity. Furthermore, preliminary studies showed that human LGL-derived clones, with B73.1⁺ OKM1⁺ phenotype had NK activity and also secreted IL-1, suggesting that these two functions may be carried out by the same cell. It has been reported that another subset(s) of LGL is capable of producing IL-2⁵. Thus, at least two functionally and phenotypically distinct subsets of LGL have been identified; our data also raise the possibility that a third subset, of OKT8⁺ LGL, may participate in the negative regulation of IL-1 production by LGL.

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- Herberman, R. B. & Ortaldo, J. R. *Science* **214**, 24–30 (1981).
- Timonen, T., Ortaldo, J. R. & Herberman, R. B. *J. exp. Med.* **153**, 569–582 (1981).
- Ortaldo, J. R., Sharrow, S. O., Timonen, T. & Herberman, R. B. *J. Immun.* **127**, 2401–2409 (1981).
- Domzig, W. & Stadler, B. M. in *NK Cells and Other Natural Effector Cells* (ed. Herberman, R. B.) 409–414 (Academic, New York, 1982).
- Kasahara, T., Djou, J. Y., Dougherty, S. F. & Oppenheim, J. J. *J. Immun.* **131**, 2329–2385 (1983).
- Djou, J. Y. *et al. J. exp. Med.* **156**, 1222–1234 (1982).
- Gery, I. & Waksman, B. H. *J. exp. Med.* **136**, 128–138 (1972).
- Luger, T. A., Stadler, B. M., Katz, S. I. & Oppenheim, J. J. *J. Immun.* **127**, 1493–1498 (1981).

- Fisher, R. I., Bostick-Burton, F., Sauder, D. N., Scala, G. & Diehl, V. *J. Immun.* **130**, 2666–2670 (1983).
- Oppenheim, J. J. & Gery, I. *Immun. Today* **3**, 113–119 (1982).
- Smith, K. A., Lachman, L. B., Oppenheim, J. J. & Favata, M. F. *J. exp. Med.* **151**, 1551–1556 (1980).
- Dempsey, R. A. *et al. J. Immun.* **129**, 2506–2510 (1982).
- Perussia, B., Starr, S., Abraham, S., Fanning, V. & Trinchieri, G. *J. Immun.* **130**, 2133–2141 (1983).
- Wands, J. R., Carlson, R. I., Schoemaker, H., Isselbacher, K. J. & Zulewsky, V. R. *Jr Proc. natn. Acad. Sci. U.S.A.* **78**, 1214–1218 (1981).
- Hanjan, S. N. S., Kearney, J. F. & Cooper, M. D. *Clin. Immun. Immunopath.* **23**, 172–188 (1982).
- Kung, P. C., Goldstein, G., Reinherz, E. L. & Schlossman, S. F. *Science* **206**, 347–349 (1979).
- Reinherz, E. L., Kung, P. C., Goldstein, G. & Schlossman, S. F. *J. Immun.* **124**, 1301–1302 (1980).
- Breard, J. M., Reinherz, E. L., Kung, P. C., Goldstein, G. & Schlossman, S. F. *J. Immun. Immunopath.* **18**, 145 (1980).
- Harada, H., Ogata, K., Kasahara, T., Shioiri-Nakano, K. & Kawai, T. *J. immun. Meth.* **59**, 189–198 (1983).
- Timonen, T. *et al. Cell. Immun.* **72**, 178–185 (1982).
- Oppenheim, J. J., Stadler, B. M., Siraganian, R. P., Mage, M. & Mathieson, B. *Fedn Proc.* **41**, 111–116 (1982).
- Gary, A. K., Daniele, R. P. & Howell, P. C. *J. Immun.* **128**, 1776–1780 (1982).
- Schmidt, J. A., Mizel, S. B., Cohen, D. & Green, I. *J. Immun.* **128**, 2177–2182 (1982).
- Farrar, W. L., Mizel, S. B. & Farrar, J. J. *J. Immun.* **124**, 1371–1377 (1980).
- Farrar, J. J. *et al. Immun. Rev.* **63**, 129–166 (1982).
- Rosenberg, S. A. & Lipsky, P. E. *J. Immun.* **126**, 1341–1345 (1981).
- Postlethwaite, A. E., Lachman, L. B., Mainardi, C. L. & Kang, A. H. *J. exp. Med.* **152**, 801–806 (1983).
- Murphy, P. A., Simon, P. L. & Willoughby, W. F. *J. Immun.* **124**, 2498–2501 (1980).
- Sztein, M. B. *et al. Cell. Immun.* **63**, 164–176 (1981).
- Mizel, S. B., Rosenstreich, D. L. & Oppenheim, J. J. *Cell. Immun.* **40**, 230–235 (1978).
- Stadler, B. M., Dougherty, S. N., Farrar, J. J. & Oppenheim, J. J. *J. Immun.* **127**, 1936–1940 (1981).

Mouse Ia antigens are receptors for lactate dehydrogenase virus

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Infection of mice with lactate dehydrogenase virus (LDV) leads to elevation of plasma lactate dehydrogenase, lifelong viraemia and perturbations of cell-mediated and humoral immune responses. The virus replicates exclusively in a restricted set of macrophages^{1,2}, but the basis for restricted cell susceptibility is unknown. By immunofluorescence techniques we have found that the per cent infected was the same as the per cent expressing antigens encoded by the I region of the major histocompatibility complex (Ia). Infection of CBA strain I-A⁺ peritoneal macrophages was blocked when cells were treated simultaneously with monoclonal antibody to I-A and I-E, but not with either antibody separately. LDV infectivity was inactivated when virus was treated with purified rat glycoprotein homologous to mouse I-A and I-E antigens. These results indicate that the receptors for LDV are I-A and I-E antigens. Selective infection of Ia-positive macrophages may have an important effect on the immunological capability of infected mice.

LDV (a strain received from Dr K. E. K. Rowson), in the form of serum taken 24 h after intraperitoneal (i.p.) infection of mice, was added to resident peritoneal macrophages of CD1 mice. Infected (antigen-containing) cells were scored by fluorescent antibody staining 10 h later. As the multiplicity of infection increased to 50 ID₅₀s (50% infectious dose) per cell, 4–10% cells became infected but this did not increase at 10,000 ID₅₀s per cell. Stueckmann *et al.*^{3,4} have obtained similar results and also noted that it became progressively more difficult to infect peritoneal macrophages when they were maintained *in vitro* for longer than 24 h. Peritoneal macrophages are known to become Ia-negative after 1–2 days in culture⁵.

Macrophages were obtained from various sites in CD1 mice and the percentage of cells supporting LDV replication and the percentage that were Ia-positive were determined (Table 1). Macrophages from all sites tested showed a close correlation between Ia positivity and susceptibility to LDV.

The expression of Ia antigens on macrophages can be regulated by prostaglandins of the E series⁶. Peritoneal macrophages

Table 1 Susceptibility to LDV infection and Ia positivity in macrophages from CD1 mice

Macrophage source	Ia-positive (%)	LDV-positive (%)
Peritoneal	5.5	4.9
Spleen	17.6	14.3
Liver	24.5	24.8
Thymus	65.2	61.0
Peripheral blood	6.7	5.9
Peritoneal macrophages from mice injected with indomethacin	25.8	23.5
Peritoneal macrophages from mice injected intraperitoneally with lymphokines	16.9	16.5

Spleen and thymus macrophages were obtained by mincing with forceps and lysing red cells with 0.83% NH_4Cl buffer. Liver macrophages were obtained by digesting with 0.05% collagenase. Peripheral blood monocytes were separated by Ficoll-Hypaque. Cells were incubated in minimal essential medium containing 10% fetal calf serum in 'ring' cultures at 37 °C with 5% CO_2 in air. After 3 h, cultures were washed vigorously to remove non-adherent cells, fixed for 10 min at 20 °C with 1% paraformaldehyde and the % Ia⁺ cells determined by indirect fluorescent antibody staining with monoclonal antibody to I-A (OX6 used at 10 $\mu\text{g ml}^{-1}$). Other cells were infected with LDV at a multiplicity of infection of 10⁴ ID₅₀s per cell. After 10–12 h, cultures were washed, dried, acetone-fixed and stained by the indirect fluorescent antibody technique for LDV antigens using mouse hyperimmune serum. The % of LDV-infected or Ia⁺ cells was determined from examination of 20–50 fields, containing 500–1,000 cells, on each of 3–6 ring cultures. Counts on each ring culture varied by less than 20%. Each type of macrophage was obtained from a separate mouse or pool of mice and in repeat experiments the per cent Ia⁺ and LDV-positive cells varied by up to twofold. In a given macrophage preparation, however, the % Ia⁺ and LDV⁺ were very similar.

were taken from mice that had received 50 μg indomethacin i.p. every 12 h for 72 h, and infected with LDV. The proportion of Ia-positive (Ia⁺) cells was 25.8% and an equivalent number of cells were infected (Table 1). The percentage of Ia⁺ cells was then increased by *in vivo* treatment with 0.1 ml of a 50% dilution of a 24-h supernatant from concanavalin A (Con A)-treated mouse spleen cell cultures; this was injected i.p. into mice every 12 h for 72 h and peritoneal cells were taken 12 h after the last

injection. 16.7% of the cells were Ia⁺ and 16.5% of the cells were infected. On the other hand, when peritoneal macrophages were maintained for 24 h *in vitro* before infection only 1–3% of the cells were Ia⁺ and infectibility decreased likewise. A double labelling experiment was carried out in which peritoneal macrophages from normal CD1 mice were infected with LDV and stained for Ia and LDV antigens with rhodamine- and fluorescein-conjugated antibody after 7 h. The percentage of Ia⁺ cells (11.0% at the initiation of infection) had decreased to 5.2% as a result of maintenance *in vitro* for 10 h; 6.1% of LDV-positive cells were now Ia-negative, but 84.6% of the remaining Ia-positive cells were LDV-positive.

The above observations show that the presence of Ia antigens in macrophages is correlated with susceptibility to LDV. This was investigated more directly by blocking infection with antibody to Ia antigens. Peritoneal macrophages from various strains of mice were treated with monoclonal antibodies that react with mouse I-A (OX6 and OX3; refs 7, 8) and with mouse I-E (H10-81.10; ref. 9), before infection with LDV. When antibody to I-A or I-E was used alone there was no influence on the virus-susceptibility of macrophages from CBA mice but there was a reduction in infection when monoclonal antibody OX6 (reacting with I-A on CBA macrophages) and antibody to I-E were added at the same time (Table 2). The monoclonal antibody OX6 does not react with BALB/c I-A, and in BALB/c macrophages the addition of monoclonal antibody to I-E fails to block LDV infectivity. I-E antigens are not expressed in H-2b, H-2f and H-2q mice¹⁰ and in macrophages from C57BL/6 (H-2b) mice, monoclonal antibody to I-A (OX3) alone blocked the infectivity of LDV. An experiment with macrophages from I-E-negative congenic mouse strains (Table 3), showed that the monoclonal antibody to I-A did not block LDV infection unless it reacted with macrophages⁷. As would be expected (data not shown), blocking was found to be less effective when a higher multiplicity of infection (500 ID₅₀s per cell) was used. Blocking of LDV infection was never complete, perhaps because large amounts of antibody are needed to block 100% of virus receptors on cells. Monoclonal antibody to mouse macrophages, or to mouse macrophage Fc receptors, failed to influence the LDV-susceptibility of peritoneal macrophages.

The results above were obtained by fluorescent antibody staining. On one occasion cultures were treated with actinomycin D, pulsed with tritiated uridine, and infected cells counted after

Table 2 Effect of monoclonal anti-Ia antibodies on the susceptibility of peritoneal macrophages to infection with LDV

Antibody dilution	C57BL/6 (<i>H</i> -2 ^b)	% Inhibition of infection							
		—	CBA (<i>H</i> -2 ^k) Anti-I-E dilutions		1:512	—	BALB/c (<i>H</i> -2 ^d) Anti-I-E dilutions		1:512
			1:128	1:256			1:128	1:256	
OX3	+	—	-2.2±4.2	2.2±8.8	11.1±6.0	—	-17.0±7.5	-13.0±5.1	-12.1±2.4
1:128	68.6±6.1*	4.5±1.3							
1:256	56.9±6.7†	6.7±2.8							
1:512	47.1±4.0†	-6.6±4.5							
OX6	—	+	73.3±1.8*	53.3±3.3*	40.0±3.5*	-21.4±5.2	—	—	—
1:128	-14.7±5.3	4.4±2.9							
1:256	-12.1±5.6	-2.2±2.1							
1:512	—	-22.2±5.1							
Anti-I-E	—	+	—	—	—	+	—	—	—
1:128	11.7±5.4	13.3±3.3							
1:256	2.0±3.9	-17.7±6.4							
1:512	-2.0±12.2	-22.2±6.2							
Anti-Fc receptor	+	—	—	—	—	—	—	—	—
1:1	-3.9±9.2	—							
Anti-macrophage	+	—							
1:50	13.1±2.3	—							

Resident peritoneal macrophages from C57BL/6, CBA and BALB/c mice were cultured in rings for 4–5 h after cell seeding. After removing non-adherent cells, macrophages were pretreated with monoclonal antibody to I-A (OX3, OX6), I-E (H10-81-10) or with a combination of anti-I-A and anti-I-E antibodies for 15 min at 20 °C. As controls, cells were treated in a similar way with rat monoclonal anti-mouse Fc receptor antibody (2-4G2), used as a culture supernatant^{17,18} or anti-mouse macrophage antibody (F4/80) used as a concentrated culture supernatant¹⁹. After three washes with phosphate-buffered saline, the cells were infected with LDV at multiplicity of infection (MOI) = 50 at 37 °C for 40 min. The cultures were re-fed with minimal essential medium supplemented with 10% fetal calf serum and incubated for 8–10 h at 37 °C in 5% CO_2 . The slides were then processed as described in Table 1, and LDV antigen-positive cells were counted in 1,000–2,000 cells on each of two rings. Per cent inhibition of infection was calculated as the difference between control and test divided by the control × 100. Each value represents the mean ± 1 s.e. from two experiments. *P* values were calculated by Student's *t*-test. Reactivity pattern of cells from different mouse strains with each monoclonal antibody was checked by indirect immunofluorescence. Anti-I-E antibody was used simultaneously with OX3 or OX6.

* *P* < 0.001; † *P* < 0.005.

Table 3 Inhibition by anti-I-A antibody of LDV infection of macrophages from I-E-negative mouse strains

OX6 dilution	B10A (4R) (H-2 ^{k/b})	% Inhibition of infection B10.M (H-2 ^f)	B10.G (H-2 ^g)	C57BL/10 (H-2 ^b)
1:128	75.0±2.1*	70.6±5.1‡	-6.3±1.2	-1.0±5.2
1:256	63.6±2.1*	55.9±11.2§	9.5±3.0	-1.0±7.8
1:512	61.4±3.5‡	8.8±1.7	-9.9±1.4	5.6±5.0
Reactivity of cells with OX6	+	+	-	-

Peritoneal macrophages from I-E-negative mouse strains were treated with monoclonal antibody to I-A (OX6), infected with LDV and the results are expressed as % inhibition of infection as shown in Table 2. The reactivity pattern of cells with this antibody is shown as described elsewhere⁷ and was confirmed by immunofluorescence staining. Each value is the mean±1 s.e. of four ring cultures.

* $P < 0.001$; † $P < 0.005$; ‡ $P < 0.01$; § $P < 0.025$.

Table 4 Inhibition of LDV infection by purified Ia antigens

Protein concentration (µg ml ⁻¹)	% Inhibition of infection		
	I-A antigen	I-E antigen	Thy 1.2 antigen
100	97.0±2.8*	87.2±2.9*	1.3±8.9
10	58.3±4.5†	66.0±13.3‡	-2.3±9.6
1	33.3±11.1§	31.9±8.0§	
0.1	16.6±13.0	2.1±10.6	

Serial 10-fold dilutions of purified rat Ia antigens (homologous to mouse I-A and I-E antigen) and mouse Thy 1.2 antigen were incubated with LDV (1×10^7 ID₅₀ ml⁻¹) for 1 h at 4 °C, then added to C57BL/6 mouse peritoneal macrophages at a calculated MOI of 20 ID₅₀ per cell. LDV antigen-positive cells were counted in 1,000–2,000 cells for each of four ring cultures 10 h later. The data are presented as described in Table 2. The antigens were prepared by 2% deoxycholate extraction of spleen cells, affinity column purification with monoclonal antibodies, elution, and removal of deoxycholate by ethanol precipitation.

* $P < 0.001$; † $P < 0.005$; ‡ $P < 0.01$; § $P < 0.05$.

autoradiography². Blocking of infection of CBA macrophages by anti-I-A plus anti-I-E antibody, but not by either antibody alone, was confirmed (data not shown).

In double-labelling experiments with fluorescein- and rhodamine-labelled antibody using rabbit antiserum to I-E and mouse monoclonal antibody to I-A, it was noted that I-A and I-E antigens were present on the same peritoneal macrophages. Rabbit anti-rat I-E antiserum plus complement was therefore used to lyse Ia-positive macrophages. With serum dilutions up to 1:100 there was a 10% reduction in cell numbers and a 90% reduction in susceptibility to LDV (data not shown).

Finally, a non-immunological method was used to show that Ia antigens are receptors for LDV. LDV was pre-treated with purified rat Ia antigens, either E or A type glycoprotein, which are homologous to mouse I-A and I-E antigens⁸, then infectivity was assayed in C57BL/6 (I-E-negative) peritoneal macrophages (Table 4). At a calculated multiplicity of 20 ID₅₀ per cell, a dose-dependent significant inhibition of infection of macrophages was seen with dilutions of either antigen down to 1 µg ml⁻¹. Murine cytomegalovirus is another virus that infects only a minority of peritoneal macrophages¹¹ but this was not influenced by treating macrophages with antibody to Ia antigens, nor by treating the virus with Ia antigens (data not shown).

If Ia antigens function as receptors for LDV, the susceptibility of a subset of macrophages is accounted for. Stueckemann *et al.*⁴ suggested that infected macrophages are eliminated, lifelong infection in mice being maintained by continuous production of fresh susceptible macrophages. This is explained if Ia-positive (virus-susceptible) macrophages are constantly generated. We have observed that the proportion of Ia-positive peritoneal macrophages is lower (1–3%) in persistently infected mice, suggesting selective loss of these cells.

Selective infection of Ia-positive, antigen-presenting cells might be expected to lead to perturbation of immune responses. If the cells that present viral antigen are at the same time the source of antigens, and are possibly damaged or destroyed, this could result in impaired immune responses to the infecting virus. Isakov *et al.*¹² reported impaired presentation of antigens by LDV-infected macrophages. Others have described enhanced antibody responses early after infection and depressed responses at a later stage, but the mechanisms are unclear¹³. Defects in cell-mediated responses have been described¹⁴, and there is so far no evidence that mice develop cell-mediated immune responses to LDV.

It might have been expected that ubiquitous antigens encoded by the major histocompatibility complex would, on occasion, be used as receptors by an infecting virus but so far there has been little evidence for this. Mouse H-2 antigens can function as receptors for Semliki Forest virus¹⁵ but these are not the only receptors because H-2-negative mouse cells can be infected with this virus¹⁶. Perhaps LDV can, on occasion, use non-Ia receptors.

The possibility that other Ia-positive cells, such as Langerhan's cells, B cells, dendritic cells and endothelial cells are also infected by LDV is being investigated.

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- Porter, D. D., Porter, H. G. & Deelake, B. B. *J. Immun.* **102**, 431–436 (1969).
- Tong, S. L., Stueckemann, J. A. & Plagemann, P. G. W. *J. Virol.* **22**, 219–227 (1977).
- Stueckemann, J. A. *et al. J. gen. Virol.* **59**, 245–262 (1982).
- Stueckemann, J. A. *et al. J. gen. Virol.* **59**, 263–272 (1982).
- Beller, D. I. & Unanue, E. R. *J. Immun.* **126**, 263–269 (1981).
- Snyder, D. S., Beller, D. I. & Unanue, E. R. *Nature* **299**, 163–165 (1982).
- McMaster, W. R. & Williams, A. F. *Eur. J. Immun.* **9**, 426–433 (1979).
- Fukumoto, T., McMaster, W. R. & Williams, A. F. *Eur. J. Immun.* **12**, 237–243 (1982).
- Pierres, M., Kourilsky, F. M., Rebouah, J. P., Dosseto, M. & Caillol, D. *Eur. J. Immun.* **10**, 950–957 (1980).
- Jones, P. P., Murphy, D. B. & McDevitt, H. O. *Immunogenetics* **12**, 312–327 (1981).
- Mims, C. A. & Gould, J. *J. gen. Virol.* **41**, 143–153 (1978).
- Isakov, N., Feldman, M. & Segal, S. *4th Int. Congr. Immun., Paris Abstr.* **11.1.09**. (1980).
- Isakov, N., Feldman, M. & Segal, S. *J. Immun.* **128**, 969–975 (1982).
- Michaelides, M. C. & Sims, E. S. *Cell Immun.* **29**, 285–294 (1977).
- Helenius, A. *et al. Proc. natn. Acad. Sci. U.S.A.* **75**, 3846–3850 (1978).
- Oldstone, M. B. A. *et al. J. Virol.* **34**, 256–265 (1980).
- Peiris, J. S. M., Gordon, S., Unkeless, J. C. & Porterfield, J. S. *Nature* **289**, 189–191 (1981).
- Unkeless, J. C. *J. exp. Med.* **150**, 580–596 (1979).
- Austyn, J. M. & Gordon, S. *Eur. J. Immun.* **11**, 805–815 (1981).

A synthetic peptide that is a bombesin receptor antagonist

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The tetradecapeptide bombesin was originally isolated from frog skin¹. Bombesin-like peptides have since been detected in mammalian gastrointestinal tract^{2,3}, brain^{3,4} and lung^{5,6}. These peptides have potent pharmacological effects on the central nervous system⁷; they cause contraction of intestinal, uterine and urinary tract smooth muscle⁸; and stimulate the release of other peptides including gastrin^{2,3,9}, cholecystokinin⁹, motilin⁹, pancreatic polypeptide⁹, neurotensin⁹, insulin^{9,10}, enteroglucagon^{9,10}, prolactin^{11,12} and growth hormone^{11,12}. Specific plasma membrane receptors for bombesin have been

demonstrated on pancreatic acinar cells¹³, brain membranes¹⁴ and pituitary cells¹⁵. Studies defining the physiological importance of bombesin have been impeded by the lack of a bombesin receptor antagonist. Here we describe experiments which demonstrate that a peptide originally described as a substance P receptor antagonist¹⁶, [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]substance P, is also a bombesin receptor antagonist. This peptide competitively inhibits the ability of bombesin to stimulate enzyme secretion from dispersed pancreatic acini, and also inhibits the action of other peptides that interact with the bombesin receptor.

A recent study of the ability of analogues of substance P (SP) to act as specific, competitive substance P receptor antagonists, showed that high concentrations of one analogue, [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP, also inhibited bombesin-stimulated amylase release¹⁷. Because there are no known competitive inhibitors of bombesin, we investigated this inhibition in more detail, and now report that [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP is a bombesin receptor antagonist.

Dispersed acini from guinea pig pancreas were prepared as described previously^{18,19}. Previous studies have shown that these acini possess specific, high-affinity plasma membrane receptors for bombesin¹³, substance P²⁰, muscarinic cholinergic agents²¹, cholecystokinin²², vasoactive intestinal peptide (VIP)^{21,23} and secretin^{21,23,24}. The bombesin receptor interacts only with bombesin and structurally related peptides^{13,21} (litorin, ranatensin, alytesin and gastrin-releasing peptide) and occupation of the bombesin receptor by any of these peptides causes mobilization of cellular calcium and stimulation of enzyme secretion^{13,25,26}.

Dispersed acini were suspended in a standard incubation solution containing 24.5 mM HEPES (pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM NaH₂PO₄, 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 2 mM glutamine, 16.5 mM glucose, 0.5 mM CaCl₂, 1.0 mM MgCl₂, 5 mM theophylline, 1% (w/v) albumin, 0.01% (w/v) trypsin inhibitor, 1% (v/v) amino acid mixture and 1% (v/v) essential vitamin mixture. The incubation solution was equilibrated with 100% O₂ and all incubations were performed with 100% O₂ as the gas phase.

Bombesin caused a 10-fold stimulation of amylase release (Fig. 1a). Detectable stimulation occurred with 0.03 nM bombesin, half-maximal stimulation with 0.16 nM bombesin and maximal stimulation with 1 nM bombesin. [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP in concentrations up to 100 µM did not stimulate amylase release but caused a parallel rightward shift in the dose-response curve for bombesin-stimulated amylase release (Fig. 1a). With increasing concentrations of [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP, the magnitude of the rightward shift was proportional to the concentration of the peptide (Fig. 1a). When the data from Fig. 1a were plotted in the form of Schild²⁷, that is, log(dose ratio - 1) versus log antagonist concentration, the values could be described by a single regression line having a slope (and 95% confidence limits) of 1.00 (0.84-1.15) (Fig. 1b). These results demonstrate that in addition to being a competitive antagonist of the substance P receptor on these acinar cells¹⁷, [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP is also a competitive antagonist of the bombesin receptor. The apparent affinity (and 95% confidence limits) of [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP for the bombesin receptor calculated for bombesin-stimulated amylase release was 2.9 (0.7-12) µM. Previously, studies have demonstrated that bombesin and litorin have affinities for the bombesin receptor of 2 nM and 40 nM, respectively¹³; therefore, the apparent affinity of [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP for the bombesin receptor is 1,500-fold lower than that for bombesin and 70-fold lower than that for litorin. The affinity of [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP for the substance P receptor on pancreatic acini has been shown to be 0.65 µM¹⁷; thus, the substance P analogue has four times greater affinity for the substance P receptor than for the bombesin receptor.

[D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP inhibited the stimulation of amylase release by peptides that interact with the bombesin

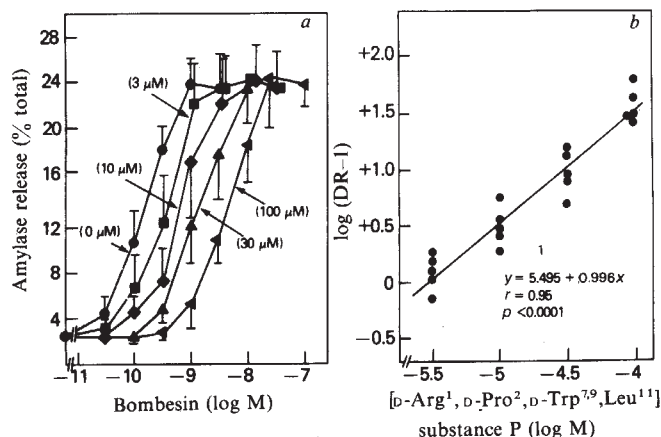


Fig. 1 Effect of various concentrations (shown in parentheses) of [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP on bombesin-stimulated amylase release. *a*, Dispersed acini from guinea pig pancreas were incubated with 0, 3, 10, 30 or 100 µM [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP with the indicated concentration of bombesin. Amylase release was measured during a 30-min incubation at 37 °C as described previously³⁰ and is expressed as the percentage of the amylase in the acini at the beginning of the incubation, that is, the percentage of the total. In each experiment each value was measured in duplicate and results are the means from five separate experiments. Vertical bars represent 1 s.d. Data from the experiments shown in *a* were plotted in the form of Schild²⁷ (*b*). The best fit to the line was calculated by least-squares analysis. DR, the dose-ratio, which is the ratio of bombesin required to give half-maximal stimulation in the presence of a given concentration of [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP to the dose required to give half-maximal stimulation in the absence of [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP.

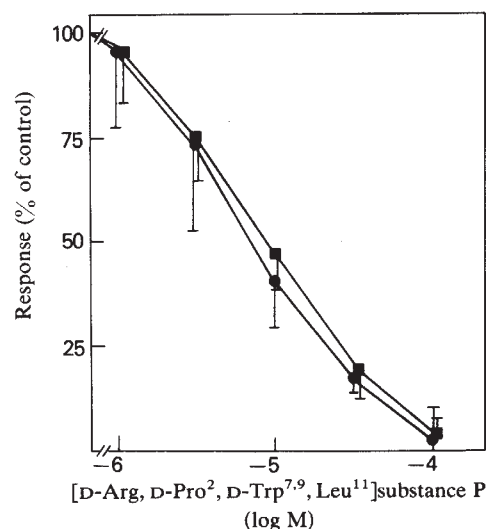


Fig. 2 Effect of [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP on bombesin-stimulated amylase release (●) and on binding of ¹²⁵I-[Tyr⁴]bombesin (○). To measure amylase release, dispersed pancreatic acini were incubated with or without 0.3 nM bombesin plus the indicated concentrations of [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP. Values for amylase release were calculated as the percentage of the increase caused by bombesin alone. To measure binding of ¹²⁵I-[Tyr⁴]bombesin, acini were incubated with 0.3 nM ¹²⁵I-[Tyr⁴]bombesin plus the indicated concentration of [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP. Saturably bound ¹²⁵I-[Tyr⁴]bombesin was determined after incubation at 37 °C for 30 min as described previously¹². Values for ¹²⁵I-[Tyr⁴]bombesin bound are calculated as the percentage of radioactivity that was saturably bound in the absence of added [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP. In each experiment each value was measured in duplicate and the results are the means of six separate experiments. Vertical bars represent 1 s.d.

receptor (bombesin, ranatensin, gastrin-releasing peptide, litorin and alytesin)^{8,13}, or by peptides that interact with the substance P receptor (substance P)²⁰ (Table 1). In contrast, [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP had no effect on stimulation caused by agents that interact with the cholecystokinin receptor (CCK-8), the cholinergic receptor (carbachol) and the vasoactive intestinal peptide receptor (VIP, secretin), or by the secretagogues 8-bromo-cyclic AMP and A23187 (Table 1).

To investigate directly the ability of [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP to interact with bombesin receptors on pancreatic acini, we measured its ability to inhibit binding of ¹²⁵I-[Tyr⁴]bombesin and its ability to inhibit bombesin-stimulated amylase release in identical experimental conditions. For each function, the dose-inhibition curves were superimposable (Fig. 2). For both inhibition of binding of ¹²⁵I-[Tyr⁴]bombesin and inhibition of bombesin-stimulated amylase release, [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP caused detectable inhibition at concentrations greater than 1 µM, half-maximal inhibition at 7.2 µM, and complete inhibition at 100 µM. These results demonstrate directly that [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP interacts with the bombesin receptor and that its ability to inhibit bombesin-stimulated amylase release can be accounted for entirely by its ability to interact with the bombesin receptor.

Table 1 Effect of [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP on the stimulation of amylase release by various pancreatic secretagogues

Secretagogue	Amylase release (% total)	
	Secretagogue alone	[D-Arg ¹ , D-Pro ² , D-Trp ^{7,9} , Leu ¹¹]SP (30 µM)
None	2.6 ± 8.1	2.7 ± 1.2
Bombesin (0.3 nM)	17.5 ± 5.0	3.8 ± 1.2*
Ranatensin (0.3 nM)	20.6 ± 4.3	6.0 ± 2.4*
Gastrin-releasing peptide (1 nM)	18.8 ± 3.0	6.0 ± 2.4*
Litorin (1 nM)	24.6 ± 5.4	5.0 ± 1.9*
Alytesin (1 nM)	17.3 ± 5.2	4.2 ± 2.8*
CCK-8 (0.1 nM)	31.7 ± 4.7	30.0 ± 3.4
Carbachol (10 µM)	31.1 ± 5.0	29.2 ± 2.3
A23187 (5 µM)	14.0 ± 2.5	14.8 ± 1.2
VIP (3 nM)	26.8 ± 2.0	27.2 ± 2.4
Secretin (0.1 µM)	26.0 ± 3.5	26.3 ± 2.7
8-bromo-cyclic AMP (1 mM)	24.0 ± 4.0	25.6 ± 1.2
Substance P (3 nM)	7.4 ± 1.4	2.4 ± 0.6*

Dispersed acini from guinea pig pancreas were incubated for 30 min at 37 °C with various secretagogues alone or with [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP. In each experiment each value was determined in duplicate and results are the means ± 1 s.d. from four separate experiments. CCK-8, the COOH-terminal octapeptide of cholecystokinin.

* Significantly different ($P < 0.01$) from the value obtained with the secretagogue alone by Student's paired *t*-test.

The apparent affinity of [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP for the bombesin receptor is much greater than that of substance P, because the latter, at concentrations as high as 30 µM, does not interact with bombesin receptors on pancreatic acini (data not shown). In contrast, the affinity of [D-Arg¹, D-Pro², D-Trp^{7,9}, Leu¹¹]SP for the substance P receptor on pancreatic acini is 2,500-fold lower than that of substance P¹⁷. Thus, modifying substance P by substitutions in positions 1, 2, 7, 9 and 11 increases the affinity of the peptide for bombesin receptors but decreases the affinity of the peptide for substance P receptors.

Several different analogues of bombesin have been tested and found not to inhibit the action of bombesin^{28,29}. Our present finding that an analogue of substance P can function as a bombesin receptor antagonist raises the possibility that appropriate modifications of the bombesin molecule may give rise to a

peptide that will function as a bombesin receptor antagonist without also interacting with the substance P receptor.

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- Anastasi, A., Ersparmer, V. & Bucci, M. *Experientia* **27**, 166-167 (1971).
- McDonald, T. J. *et al. Biochem. biophys. Res. Commun.* **90**, 227-233 (1979).
- Walsh, J. A., Wong, H. C. & Dockray, G. J. *Fedn Proc.* **38**, 2315-2319 (1979).
- Moody, T. W. & Pert, C. B. *Biochem. biophys. Res. Commun.* **90**, 7-14 (1979).
- Wharton, J. *et al. Nature* **273**, 769-770 (1978).
- Ghatei, M. A. *et al. Endocrinology* **111**, 1248-1254 (1982).
- Tache, Y. & Brown, M. *Trends Neurosci.* **5**, 431-433 (1982).
- Ersparmer, V. & Melchiorri, P. *Pure appl. Chem.* **35**, 463-494 (1973).
- Ghatei, M. A. *et al. J. clin. Endocr. Metab.* **54**, 980-985 (1982).
- Kaneto, A., Kaneko, T., Nakaya, S., Kajinuma, H. & Kosaka, K. *Metabolism* **27**, 549-553 (1978).
- Rivier, C., Rivier, J. & Vale, W. *Endocrinology* **102**, 519-522 (1978).
- Westendorf, J. M. & Schonbrunn, A. *Endocrinology* **110**, 352-358 (1982).
- Jensen, R. T., Moody, T. W., Pert, C. B., Rivier, J. E. & Gardner, J. D. *Proc. natn. Acad. Sci. U.S.A.* **75**, 6139-6143 (1978).
- Moody, T. W., Pert, C. B., Rivier, J. & Brown, M. R. *Proc. natn. Acad. Sci. U.S.A.* **75**, 5372-5376 (1978).
- Westendorf, J. M. & Schonbrunn, A. *J. biol. Chem.* **258**, 7527-7535 (1983).
- Lundberg, J. M., Saria, H., Brodin, E., Rosell, S. & Folkers, K. *Proc. natn. Acad. Sci. U.S.A.* **80**, 1120-1125 (1983).
- Jensen, R. T. *et al. Biochim. Biophys. Acta* (in the press).
- Peikin, S. R., Rottman, A. J., Batzri, S. & Gardner, J. D. *Am. J. Physiol.* **235**, E743-E749 (1978).
- Jensen, R. T., Lemp, G. F. & Gardner, J. D. *J. biol. Chem.* **257**, 5554-5559 (1982).
- Jensen, R. T. & Gardner, J. D. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5679-5683 (1979).
- Jensen, R. T. & Gardner, J. D. *Fedn Proc.* **40**, 2486-2496 (1981).
- Jensen, R. T., Lemp, G. F. & Gardner, J. D. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2079-2083 (1980).
- Christophe, J. P., Conlon, T. P. & Gardner, J. D. *J. biol. Chem.* **251**, 4629-4634 (1976).
- Jensen, R. T. *et al. Am. J. Physiol.* **245**, G186-G195 (1983).
- Iwatsuki, N. & Petersen, O. H. *J. clin. Invest.* **61**, 41-46 (1978).
- Deschodt-Lanckman, M., Robberecht, P., DeNeef, P., Lammens, M. & Christophe, J. *J. clin. Invest.* **58**, 891-898 (1976).
- Schild, H. O. *Br. J. Pharmac.* **4**, 277-280 (1949).
- Rivier, J. & Brown, M. *Biochemistry* **17**, 1766-1771 (1978).
- Marki, W., Brown, M. & Rivier, J. E. *Peptides* **2**, Suppl. 2, 169-177 (1981).
- Gardner, J. D. & Jackson, M. J. *J. Physiol., Lond.* **270**, 439-454 (1977).

The second messenger linking receptor activation to internal Ca release in liver

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The increase in cytosolic [Ca²⁺] induced by Ca-mobilizing hormones in liver is mainly due to release of Ca from intracellular stores¹⁻⁷. For Ca to be released from internal sites a messenger must be formed at the plasma membrane which diffuses into the cytosol to signal Ca release from the intracellular organelles^{2,3,7,8}. One of the first actions of these hormones is to cause breakdown of the polyphosphoinositides to form soluble inositol phosphates⁹⁻¹¹. Some evidence for the idea that these substances could be the second messenger¹⁰ has been obtained in pancreatic acinar cells¹². Here we have found that hormone activation of hepatocytes causes rapid breakdown of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] to form inositol trisphosphate (InsP₃). When applied to permeabilized hepatocytes, InsP₃ releases Ca from non-mitochondrial ATP-dependent pools. This suggests that InsP₃ could be the messenger linking Ca-mobilizing receptor activation to intracellular Ca release in liver.

Isolated hepatocytes were prepared by collagenase digestion of the livers of male guinea pigs and used either intact or after

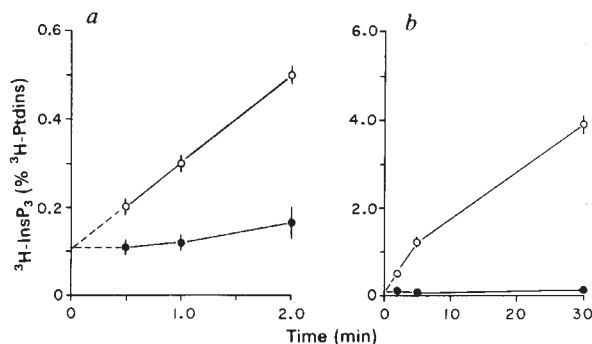


Fig. 1 Formation of ^3H -InsP₃ in guinea pig hepatocytes over 0–2 min (a) or 0–30 min (b). Freshly prepared guinea pig hepatocytes¹⁷ were incubated in Eagle's medium with 2% bovine serum albumin (BSA) with $5\ \mu\text{Ci ml}^{-1}$ (three experiments) or $20\ \mu\text{Ci ml}^{-1}$ (three experiments) of ^3H -inositol, and containing $0.1\ \mu\text{M}$ angiotensin II for 60 min. The cells were washed by slow centrifugation and resuspended in Eagle's medium with 2% BSA. After 5 min, $10\ \text{mM}$ LiCl was added to block inositol 1-phosphate phosphatase²⁴. In two preliminary experiments, LiCl had no discernible effect on ^3H -InsP₃ accumulation. Five minutes later, adrenaline ($0.1\ \text{mM}$, with $10\ \mu\text{M}$ (+)propranolol) was added (○); control, ●. Aliquots of the suspension were taken at various times as indicated, and were rapidly precipitated with 4.5% perchloric acid. The pH of the supernatant was adjusted to 8–9 with sodium borate buffer and radioactive fractions associated with specific inositol phosphates were separated by anion exchange as described previously^{14,20}. For each experiment, total lipid radioactivity (almost exclusively PtdIns) was determined in two chloroform-methanol extracts, one taken just before the addition of adrenaline and one just after the last sample was taken. The radioactivity in these samples did not change significantly over the course of the experiments, nor did total tissue content of PtdIns, determined as described previously²¹. For each experiment, the cpm of ^3H -InsP₃ was expressed as a percentage of the cpm of ^3H -PtdIns (that is, the chloroform-methanol extract). In the $5\ \mu\text{Ci ml}^{-1}$ experiments, samples were taken at 5 and 30 min and PtdIns radioactivity averaged $84,000 \pm 9,000$ c.p.m. per mg DNA; in the $20\ \mu\text{Ci ml}^{-1}$ experiments, samples were taken at 0.5, 1, 2 and 30 min and PtdIns radioactivity averaged $544,000 \pm 30,000$ c.p.m. per mg DNA. All of the data points in Fig. 1 are thus means ($\pm$ s.e.m.) of three determinations except at $t = 30$ min which is the mean ($\pm$ s.e.m.) of six experiments. In the latter case, the three experiments with $5\ \mu\text{Ci ml}^{-1}$ ^3H -inositol gave a value of $4.1 \pm 0.3\%$ while the $20\ \mu\text{Ci ml}^{-1}$ experiments gave a value of $3.7 \pm 0.3\%$, the two not differing significantly.

permeabilization of their plasma membranes with saponin, as previously described¹³. The intact hepatocytes were incubated in Eagle's medium, while the permeable cells were incubated in a medium with high $[\text{K}^+]$, low $[\text{Na}^+]$, ATP ($1.5\ \text{mM}$) and an ATP-regenerating system (see Fig. 2 legend for details). Ca-EGTA buffers were used to give free Ca^{2+} concentrations of either $180\ \text{nM}$ or $3.3\ \mu\text{M}$ (ref. 13). Formation of ^3H -inositol phosphates by hepatocytes preincubated with ^3H -inositol was measured as described previously¹⁴.

Intact guinea pig hepatocytes showed a rapid increase in radioactivity associated with InsP₃ on α -adrenoceptor activation (Fig. 1). To test the hypothesis that InsP₃ is capable of releasing internal Ca we used a permeabilized hepatocyte preparation the characteristics of which have been described in detail elsewhere¹³. In experiments summarized in Fig. 2a, permeable hepatocytes were incubated in a medium with $[\text{Ca}^{2+}]$ buffered to $180\ \text{nM}$ in the presence of tracer quantities of ^{45}Ca . This value for $[\text{Ca}^{2+}]$ was chosen because in these conditions the levels of phosphorylase α , the uptake of ^{45}Ca and the content of ^{40}Ca in saponin-treated cells were found to be similar to those in intact hepatocytes. We therefore concluded that the cytosolic $[\text{Ca}^{2+}]$ in an intact hepatocyte is close to $180\ \text{nM}$ ¹³. Recent estimates of intracellular $[\text{Ca}^{2+}]$ in rat¹⁵ and guinea pig¹³ hepatocytes by using the intracellular Ca-indicator 'quin-2' (ref. 16) confirm this estimate.

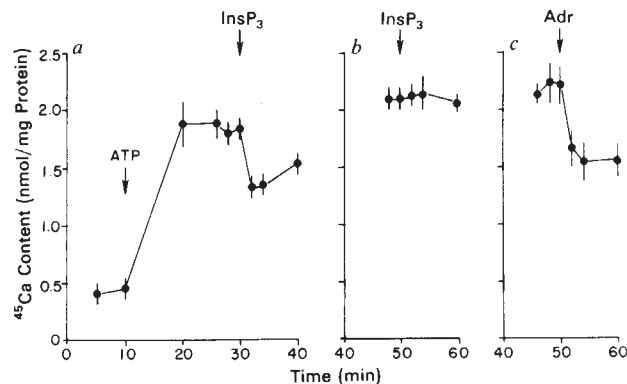


Fig. 2 a, The effect of InsP₃ ($5\ \mu\text{M}$) on the ^{45}Ca content of saponin-treated hepatocytes. b, InsP₃ ($5\ \mu\text{M}$) had no effect on the ^{45}Ca content of normal guinea pig hepatocytes; c, adrenaline ($5\ \mu\text{M}$) in the presence of $10\ \mu\text{M}$ (+)propranolol caused a net loss of ^{45}Ca from normal hepatocytes. In b and c the normal hepatocytes were loaded with ^{45}Ca for ~ 45 min (which is long enough to allow most of the intracellular Ca to exchange¹³).

Methods: At time zero ^{45}Ca was added to a suspension of saponin-treated hepatocytes incubated in a cytoplasmic-type medium¹³ of the following composition (mM): NaCl, 20.0; KCl, 100; MgSO₄, 5.0; NaH₂PO₄, 0.96; NaHCO₃, 25; EGTA, 1.0, at pH 7.2 and 37°C . The medium also contained albumin (2%), antimycin ($10\ \mu\text{M}$) and an ATP-regenerating system consisting of creatine phosphate (5 mM) and creatine phosphokinase ($5\ \text{U ml}^{-1}$). The $[\text{Ca}^{2+}]$ was set at $180\ \text{nM}$ which is believed to be $[\text{Ca}^{2+}]_i$ in a normal unstimulated guinea pig hepatocyte¹³. At 10 min, ATP ($1.5\ \text{mM}$) was added and there was a rapid uptake of ^{45}Ca by the cells, which reached steady state by 20 min. At this $[\text{Ca}^{2+}]$ the ATP-dependent ^{45}Ca uptake into the saponin-treated cells was insensitive to a combination of the mitochondrial poisons 2,4-dinitrophenol ($0.5\ \text{mM}$) and oligomycin ($10\ \mu\text{M}$) and is believed to be taken up mainly into the endoplasmic reticulum¹³. At 30 min, InsP₃ ($5\ \mu\text{M}$ in H₂O) was added and there was a loss of $\sim 25\%$ or $\sim 0.5\ \text{nmol per mg protein}$ of the cell ^{45}Ca content. An equivalent volume of H₂O alone had no effect on the ^{45}Ca content (not shown). ^{45}Ca content was determined in all experiments by centrifugation through ice-cold sucrose and EGTA as described previously²⁵. Data were averaged ($n = 5$ in a, 3 in b and 4 in c) and the bars represent the s.e.m. InsP₃ (specifically inositol (1,4,5)trisphosphate) was prepared from human red blood cell ghosts essentially by the method of Downes *et al.*²⁶, the only major modification being that the rejuvenation of the red blood cells before lysis was carried out in a chloride-free medium (the chloride being replaced by citrate^{26,27}). InsP₃ was separated on Dowex formate columns¹⁴, and desalted by elution from a Dowex chloride column by $1\ \text{M}$ LiCl. After addition of $100\ \mu\text{g}$ mannitol, the extract was dried *in vacuo*, before removal of the LiCl with ethanol. Some of the InsP₃ was further purified by high-voltage ionophoresis in pyridine/acetic acid (pH 3.6) for 1.5 h at $40\ \text{V cm}^{-1}$. The InsP₃ was eluted from the paper, and the adjacent strip of paper was also eluted as a control. For experiments not shown here, but described in the text, inositol 1,4-bisphosphate [Ins(1,4)P₂] was prepared from ox brain phosphatidylinositol 4-phosphate by strong alkaline hydrolysis, and purified by paper chromatography¹⁴. Inositol 1-phosphate (Ins1P) and inositol 1,2-cyclic phosphate were prepared enzymatically from yeast phosphatidylinositol and purified by ionophoresis^{28,29}.

On addition of ATP, the permeable cells accumulated ^{45}Ca rapidly to a level of about $2\ \text{nmol per mg protein}$ (Fig. 2a). Once a steady state had been achieved the addition of $5\ \mu\text{M}$ InsP₃ to these permeable cells caused a rapid (< 2 min) net release of about $0.5\ \text{nmol per mg protein}$ of cell-associated ^{45}Ca . This fairly closely approximates the quantity of Ca initially released from intact guinea pig hepatocytes by α -adrenergic stimuli (Fig. 2c and ref. 17). In two experiments (not shown), $5\ \mu\text{M}$ of the InsP₃ which was purified by high-voltage ionophoresis (see Fig. 2 legend) induced a similar release of ^{45}Ca from the saponin-treated cells, but the eluate from the adjacent strip of paper had no effect. However, $5\ \mu\text{M}$ InsP₃ did not cause ^{45}Ca release from intact hepatocytes (Fig. 2b), nor

did Ca-mobilizing hormones release Ca from permeable cells (not shown). InsP_3 had no effect on the ATP-independent ^{45}Ca associated with the permeable cells. Inositol 1,2-cyclic phosphate (5 μM) (previously proposed as a second messenger¹⁸), $\text{Ins}(1,4)\text{P}_2$ (up to 10 μM), $\text{Ins}1\text{P}$ (5 μM) and inositol (5 μM) had no effect on the ^{45}Ca accumulated in an ATP-dependent manner by permeable hepatocytes (not shown).

Figure 3 shows the concentration dependence of the response of the permeable cells to InsP_3 in causing the net release of ^{45}Ca which had been accumulated in an ATP-dependent manner. The EC_{50} value for this effect was 0.22 μM , and it was maximal at 5 μM .

It has previously been demonstrated that with $[\text{Ca}^{2+}]$ in the medium buffered to 180 nM, uptake of ^{45}Ca and cellular content of total Ca in permeable hepatocytes are unaffected by the mitochondrial poisons 2,4-dinitrophenol and oligomycin¹³. The ^{45}Ca released by InsP_3 (Fig. 2a) must therefore come from ATP-dependent stores other than the mitochondria, the predominant component of which is likely to be the endoplasmic reticulum³. To determine whether InsP_3 is also capable of releasing Ca from the mitochondria, the experiment in Fig. 2a was repeated but with the permeable cells suspended in a medium with $[\text{Ca}^{2+}]$ buffered to 3.3 μM . In these conditions a considerably larger quantity of ^{45}Ca accumulates (Fig. 4). The uptake of this ^{45}Ca can be largely blocked by dinitrophenol and oligomycin and is therefore presumed to be primarily mitochondrial¹³. As shown in Fig. 4, 5 μM InsP_3 produced no significant release of ^{45}Ca from the cells when $[\text{Ca}^{2+}]$ in the medium was 3.3 μM , and we conclude that mitochondrial Ca is not released by InsP_3 , at least in these conditions and with these concentrations of Ca^{2+} and InsP_3 . The release of the 0.5 nmol per mg protein of ^{45}Ca from the endoplasmic reticulum pool by InsP_3 , observed at 180 nM $[\text{Ca}^{2+}]$ (Fig. 2a), would not be statistically detectable here because of the higher ^{45}Ca content.

These findings comprise strong evidence that InsP_3 could function as a second messenger linking hormone receptor activation to internal calcium release in the liver. An important question is whether the intracellular level of InsP_3 is elevated sufficiently during hormonal activation for it to perform this Ca-mobilizing function. Although there is no adequately sensitive and specific method for measuring the chemical levels of inositol phosphates, they can be estimated if an assumption is made regarding specific radioactivities. Since the monoester phosphates of the polyphosphoinositides turn over much more rapidly than other parts of the molecules (refs 8, 19 and unpublished observations), it is reasonable to assume that in cells labelled with ^3H -inositol the specific radioactivities of the three phosphoinositides and thus also their breakdown products, the inositol phosphates, will all be similar²⁰. In each of the experiments shown in Fig. 1, therefore, the radioactivity associated with phosphatidylinositol (PtdIns) (which did not change appreciably during the experiments and was not significantly altered by hormones) was measured and the radioactivity associated with InsP_3 expressed as a percentage of that value. From these data, and the knowledge that guinea pig hepatocytes contain 37.1 ± 2.7 ($n=5$) nmol per mg protein (assayed as described previously²¹) of PtdIns, the cellular content of InsP_3 could be calculated. Assuming that hepatocytes contain 2.2 μl cell water per mg protein⁶, the net increase in cellular InsP_3 induced by a maximal concentration of adrenaline was estimated to be about 16 μM at 30 s, 32 μM at 1 min and 53 μM at 2 min. As the α -adrenoceptor-induced release of ^{45}Ca from isolated intact guinea pig hepatocytes is not maximal until 2 min (see Fig. 2 and ref. 17), the InsP_3 would seem to be formed rapidly enough to act as a second messenger.

When applied to permeable cells in concentrations even lower than these estimated values, and with free $[\text{Ca}^{2+}]$ buffered to a level similar to that in the cytosol, InsP_3 induced the release of Ca from an intracellular store. Further, the quantity of Ca was similar to that released from the intracellular stores of intact hepatocytes by hormones. These results also support the view¹³ that the primary source of hormone-sensitive Ca in the

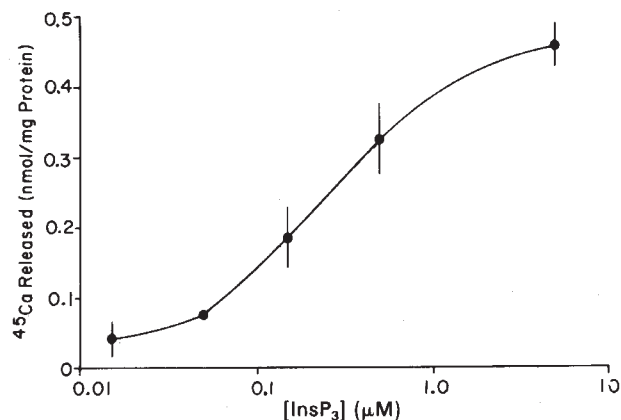


Fig. 3 The concentration-effect relationship for the ability of InsP_3 to release ^{45}Ca from permeable hepatocytes. Experiments were carried out as described for Fig. 2a and the amount of ^{45}Ca released was determined at 2 min by which time the effect was maximal. ^{45}Ca contents were determined by the rapid dilution of 100- μl aliquots of cell suspension in 10 ml of ice-cold isotonic sucrose containing 4 mM EGTA and tracer amounts of ^3H -sucrose for determination of trapped volume. The sample was then filtered rapidly through GF/C filters, washed with 10 ml of ice-cold isotonic sucrose, and the filters were counted for radioactivity. Data were averaged and the bars indicate s.e.m. for between three and five observations except at 5 μM where $n=13$ and which includes data for which the ^{45}Ca content was determined by centrifugation²⁵ (from Fig. 2a).

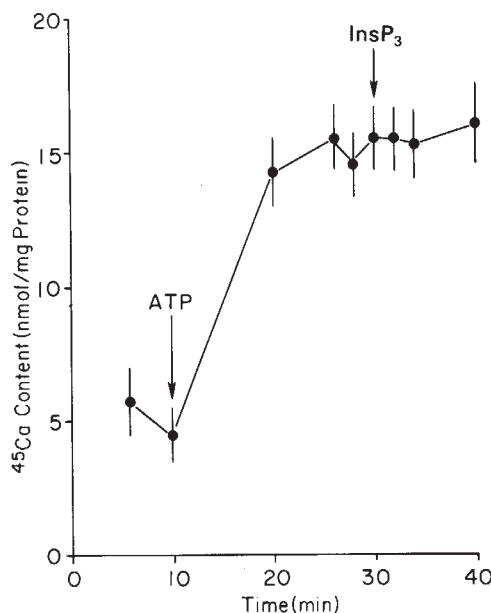


Fig. 4 Effect of InsP_3 (5 μM) on the ^{45}Ca content of saponin-treated guinea pig hepatocytes. The saponin-treated hepatocytes were incubated as described for Fig. 2a except that the $[\text{Ca}^{2+}]$ of the cytoplasmic-type medium was increased to 3.3 μM , a concentration at which there is substantial 2,4-dinitrophenol (0.5 mM)- and oligomycin (10 μM)-sensitive ^{45}Ca uptake, presumably by the mitochondria¹³. The ATP-dependent ^{45}Ca uptake was much greater than in Fig. 2a and as the endoplasmic reticulum mechanism saturates at about 3 nmol per mg protein¹³, most of the uptake is ATP-dependent uptake into mitochondria. Application of InsP_3 had no effect on the ^{45}Ca content of the cells. Data were averaged and the bars indicate s.e.m. of three observations.

liver is a non-mitochondrial pool, perhaps the endoplasmic reticulum.

In the liver, as in many other tissues, agonist-induced release of intracellular Ca is accompanied by an increased membrane permeability to Ca (ref. 6). Following inositol lipid breakdown, the resulting diacylglycerol is rapidly phosphorylated to phosphatidic acid which it has been suggested may mediate Ca entry by functioning as a Ca-ionophore (ref. 22 but see ref. 23).

Although not addressed specifically by these studies, the possibility must now be considered that InsP_3 could also regulate plasma membrane permeability to Ca by the same, as yet unknown, mechanism involved in the release of intracellular Ca.

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1. Rasmussen, H. *Calcium and cAMP as Synaptic Messengers* (Wiley, New York, 1981).
2. Exton, J. H. in *Adrenoceptors and Catecholamine Action Part A* (ed. Kunos, G.) 117-129 (Wiley-Interscience, New York, 1981).
3. Williamson, J. R., Cooper, R. H. & Hoek, J. B. *Biochim. biophys. Acta* **639**, 243-295 (1981).
4. Burgess, G. M., Claret, M. & Jenkinson, D. H. *Nature* **279**, 544-546 (1979).
5. Poggioli, J., Berthoin, B. & Claret, M. *FEBS Lett.* **115**, 243-246 (1980).
6. DeWitt, L. M. & Putney, J. W. Jr *J. Physiol., Lond.* **346**, 395-407 (1984).
7. Reinhart, P. H., Taylor, W. M. & Bygrave, F. L. *Biochem. J.* **208**, 619-630 (1982).
8. Michell, R. H. *Biochim. biophys. Acta* **415**, 81-147 (1975).
9. Kirk, C. J., Creba, J. A., Downes, C. P. & Michell, R. H. *Biochem. Soc. Trans.* **9**, 377-379 (1981).
10. Berridge, M. J. *Biochem. J.* **212**, 849-858 (1983).
11. Abdel-Latif, A. A., Akhtar, R. A. & Hawthorne, J. N. *Biochem. J.* **162**, 61-73 (1977).
12. Streb, H., Irvine, R. F., Berridge, M. J. & Schulz, I. *Nature* **306**, 67-68 (1983).
13. Burgess, G. M., McKinney, J. S., Fabbio, A., Leslie, B. A. & Putney, J. W. Jr *J. Biol. Chem.* **258**, 5716-5725 (1983).
14. Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P. & Irvine, R. F. *Biochem. J.* **212**, 473-482 (1983).
15. Charest, R., Blackmore, P. F., Berthoin, B. & Exton, J. H. *J. Biol. Chem.* **258**, 8769-8773 (1983).
16. Tsein, R. Y., Pozzan, T. & Rink, T. J. *J. Cell Biol.* **94**, 325-334 (1982).
17. Burgess, G. M., Claret, M. & Jenkinson, D. H. *J. Physiol., Lond.* **317**, 67-90 (1981).
18. Michell, R. H. & Lapetina, E. G. *Nature new Biol.* **240**, 258-260 (1972).
19. Downes, P. & Michell, R. H. *Cell Calcium* **3**, 467-502 (1982).
20. Aub, D. L. & Putney, J. W. Jr *Life Sci.* (in the press).
21. Weiss, S. J. & Putney, J. W. Jr *Biochem. J.* **194**, 463-468 (1981).
22. Putney, J. W. Jr *Life Sci.* **29**, 1183-1194 (1981).
23. Holmes, R. P. & Yoss, N. L. *Nature* **305**, 637-638 (1983).
24. Berridge, M. J., Downes, C. P. & Hanley, M. R. *Biochem. J.* **206**, 587-595 (1982).
25. Poggioli, J. & Putney, J. W. Jr *Pflügers Arch. ges. Physiol.* **392**, 239-243 (1982).
26. Downes, C. P., Mussat, M. C. & Michell, R. H. *Biochem. J.* **203**, 169-177 (1982).
27. Irvine, R. F., Letcher, A. J. & Dawson, R. M. C. *Biochem. J.* (submitted).
28. Irvine, R. F., Hemington, N. & Dawson, R. M. C. *Biochem. J.* **176**, 475-484 (1978).
29. Irvine, R. F., Letcher, A. J. & Dawson, R. M. C. *Biochem. J.* **178**, 497-500 (1979).

Cyclic nucleotides control a system which regulates Ca^{2+} sensitivity of platelet secretion

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Cellular responses to extracellular signals are mediated by changes in the intracellular concentrations of one or more second messengers¹. In platelets, inhibitory agonists increase intracellular cyclic-3',5'-AMP ([cyclic AMP]; (refs 2, 3)) whereas excitatory agonists increase $[\text{Ca}^{2+}]_i$ and/or [1,2-diacylglycerol]; (refs 4-9), and in some cases decrease [cyclic AMP]; (refs 10, 11). Both activation and inhibition of platelet responses have been attributed to an increase in [cyclic-3',5'-GMP]; (refs 8, 12). The activity of protein kinase C, which is associated with the platelet secretory response, is increased by both 1,2-diacylglycerol and Ca^{2+} (refs 4, 7, 8). The role of cyclic AMP may involve either inhibition of Ca^{2+} mobilization to the cytosol¹³ or stimulation of intracellular Ca^{2+} uptake¹⁴, and in addition inhibition of 1,2-diacylglycerol formation^{15,16}. The relationship between cyclic-3',5'-GMP (cyclic GMP) and other second messengers in platelet activation has not been defined. Using platelets made permeable by exposure to an intense electric field^{17,18}, we demonstrate here modulation of the Ca^{2+} sensitivity of platelet secretion by thrombin, and by 12-O-tetradecanoylphorbol-13-acetate (TPA) and 1-oleyl-2-acetylglucol (OAG), both potent activators of protein kinase C. The effect of thrombin is selectively modified by cyclic GMP and cyclic AMP. The response to OAG and TPA is also modulated by cyclic AMP but to a much lesser extent.

An increase in $[\text{Ca}^{2+}]_i$ has previously been shown to induce ^{14}C -serotonin secretion from permeabilized platelets¹⁹. Addi-

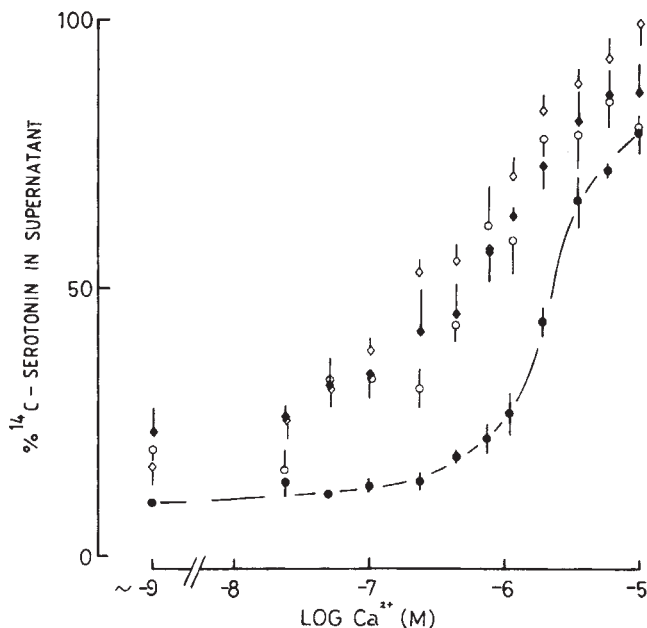


Fig. 1 The Ca^{2+} sensitivity of ^{14}C -serotonin secretion from platelets in the absence (●) and in the presence of thrombin (◇), OAG (○) or TPA (◆). Human platelets were prepared in the presence of 0.1 mM acetylsalicylate as described previously¹⁸, and were suspended in medium containing (in mM): glycine, 280; K-glutamate, 10; ATP, 5; Mg-acetate, 7; PIPES, 20; EGTA, 1; pH 6.7. The platelets were rendered permeable by 10 exposures to an intense electric field (20 kV cm^{-1} , $\tau \sim 30 \mu\text{s}$)¹⁷. After incubation for 15 min at 22°C , 1-ml aliquots of the suspension ($\sim 2 \times 10^8$ cells ml^{-1}) were added to Ca-EGTA buffers and exactly 40 s later 0.2-ml aliquots of these Ca-challenged cells were added to 0.02 ml of thrombin, OAG, TPA or a control suspending medium. The final concentrations of EGTA, thrombin, OAG and TPA in the cell suspension were 16 mM, 0.5 U ml^{-1} , $30 \mu\text{g ml}^{-1}$ and 10 ng ml^{-1} , respectively. After incubation for 5 min at 22°C the platelets were removed by centrifugation at $8,000g$ for 2 min and 0.05-ml aliquots of the supernatant fraction assayed for ^{14}C content by liquid scintillation counting. The time taken to challenge the cells with all the Ca buffers was 10 min, with the order of Ca^{2+} challenges being random. The ordinate shows the amount of ^{14}C in the supernatant fraction expressed as a percentage of the total ^{14}C measured by counting aliquots of the cell suspension. The data points refer to the mean of four determinations with the bar showing the s.e.m. OAG and TPA were prepared as stock solutions (60 mg ml^{-1} and $10 \mu\text{g ml}^{-1}$, respectively) in dimethyl sulphoxide (DMSO), dispersed in the suspending medium by sonication under N_2 for 5 s, then diluted appropriately in that medium. The final concentration of DMSO in the cell suspension was 0.1% (v/v); control experiments showed that this level of DMSO had no effect on the secretory response and that addition of thrombin, OAG and TPA caused no significant increase in the extent of cellular lysis as measured by release of lactate dehydrogenase. Ca^{2+} concentrations were calculated using equilibrium constants of $10^{-6.127}\text{M}$ (Ca-EGTA), $10^{-1.362}\text{M}$ (Mg-EGTA), $10^{-3.50}\text{M}$ (Ca-ATP) and $10^{-3.64}\text{M}$ (Mg-ATP)³⁴. The EC_{50} value for Ca^{2+} is $2.0 \mu\text{M}$ in the absence of other additions and $0.3\text{--}0.5 \mu\text{M}$ in the presence of 0.5 U ml^{-1} thrombin, 10 ng ml^{-1} TPA or $30 \mu\text{g ml}^{-1}$ OAG.

tion of thrombin or a synthetic activator of protein kinase C such as OAG²⁰ or TPA^{21,22}, increases the sensitivity of the secretory response to Ca^{2+} by shifting the dose-response curve to the left (Fig. 1). In addition, thrombin, TPA and OAG cause a small increase in the extent of secretion at 1 nM Ca^{2+} . Although this increase could indicate the existence of a secretory mechanism which is not controlled by Ca^{2+} (refs 6, 7), its contribution to the overall secretory response is minimal in the conditions used here. Since both TPA and OAG mimic the effect of thrombin (Fig. 1), these data strongly support the proposed role of protein kinase C in amine storage granule secretion^{7,8}. A similar increase in the sensitivity of the secretory

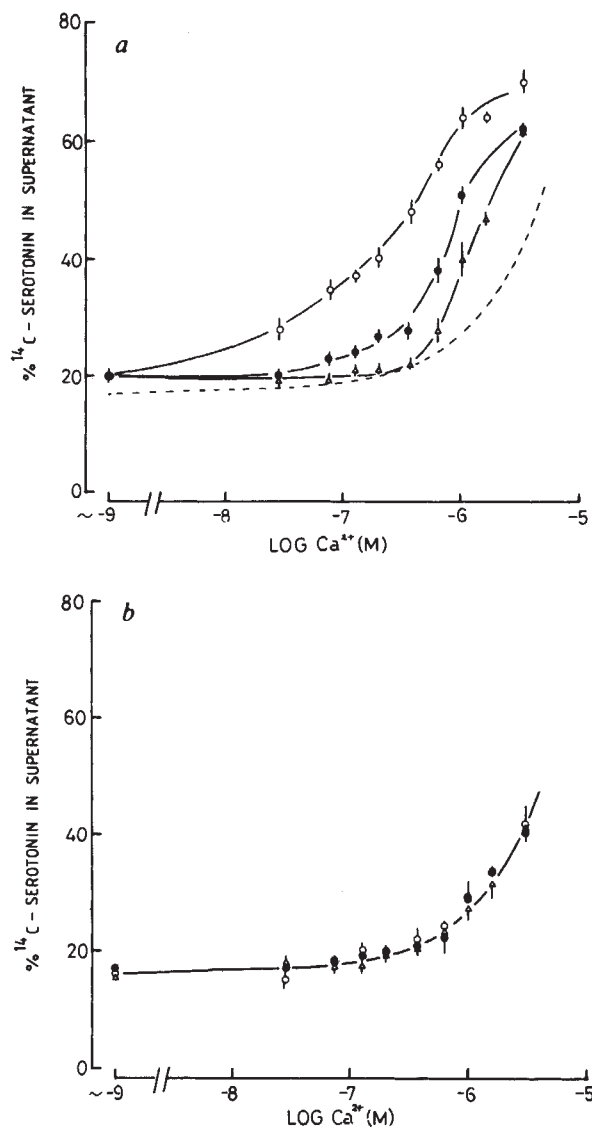


Fig. 2 The effect of cyclic AMP and cyclic GMP on the Ca^{2+} sensitivity of ^{14}C -serotonin release in the presence (a) or absence (b) of thrombin. Platelets were prepared and rendered permeable as described for Fig. 1 except that the suspending medium contained 1 mM ATP, 3 mM Mg-acetate, 0.5 mM EGTA and at a pH of 6.6. After incubation for 25 min at 22 °C, aliquots of the permeabilized suspension ($\sim 4 \times 10^8$ cells ml^{-1}) were incubated for 5 min at 22 °C with 0.2 μM 3-isobutyl-1-methylxanthine (IBMX) and 1 mM bis(*O*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) either alone (●), in the presence of 20 μM cyclic AMP (△) or in the presence of 20 μM cyclic GMP (○). 0.1-ml aliquots of these suspensions were added to 0.1 ml of the suspending medium containing Ca-EGTA buffers either alone (b) or in the presence of thrombin (a). The final concentrations of EGTA and thrombin were 20 mM and 0.5 U ml^{-1} , respectively. The system was incubated for 5 min at 22 °C, centrifuged and the supernatant fraction assayed for ^{14}C content as described for Fig. 1. Data points refer to the mean of four determinations with the error bars showing the s.e.m. When Ca-EGTA buffers were added to the cell suspension containing BAPTA, the pH increased from 6.6 to 6.7. The Ca^{2+} concentrations stated above were calculated from the Ca-EGTA stability constants at these pHs³⁴, and from the stability constants for Ca-BAPTA and Mg-BAPTA of $10^{-6.959}$ M and $19^{-1.77}$ M, respectively³⁵. The curve drawn through the data points of cells incubated in the absence of thrombin (b) is also included as a dashed line in a to facilitate comparison with the data obtained in the presence of thrombin $\pm$ the cyclic nucleotides. The EC_{50} values for Ca^{2+} obtained from these data are 2.0 μM (no additions), 0.5 μM (in the presence of 0.5 U ml^{-1} thrombin), 1.2 μM (in the presence of 0.5 U ml^{-1} thrombin + 20 μM cyclic AMP) and 0.15 μM (in the presence of 0.5 U ml^{-1} thrombin + 20 μM cyclic GMP).

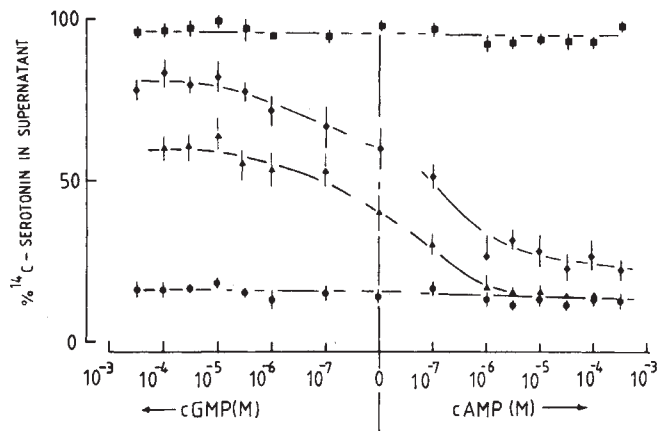


Fig. 3 Dose-response curves for the effects of cyclic AMP and cyclic GMP on ^{14}C -serotonin secretion mediated by thrombin in the presence of four fixed Ca^{2+} concentrations. Platelets were prepared and suspended in the medium described for Fig. 1 except that 1 mM BAPTA was added instead of 1 mM EGTA, and the pH of the medium was 6.6. The cells were rendered permeable as described for Fig. 1, then incubated for 8–10 min at 22 °C and at a cell density of 4×10^8 cells ml^{-1} in the presence of 5 μM IBMX and at concentrations of either cyclic AMP or cyclic GMP as shown. 0.1-ml aliquots of these suspensions were then added to 0.1 ml of the suspending medium containing thrombin and Ca-EGTA buffers to give final Ca^{2+} concentrations (calculated) of ~ 1 nM (●), 0.15 μM (▲), 0.5 μM (◆) and 5.0 μM (■). The final concentrations of thrombin and EGTA were 0.7 U ml^{-1} and 7.7 mM, respectively. After incubation for 5 min at 22 °C the platelets were removed and the supernatant fraction assayed for ^{14}C content as described for Fig. 1. The data points are the mean of four determinations, with error bars showing the s.e.m. The final Ca^{2+} concentrations were calculated as described for Fig. 2. No significant enhancement of the response was observed at 0.15 μM or 0.5 μM Ca^{2+} when 20 μM cyclic-2'3'-GMP was used in place of cyclic-3'5'-GMP.

response to Ca^{2+} has been observed on addition to TPA to permeabilized adrenal medullary cells²³. The data also explain the previously reported²⁴ difference in secretory behaviour between intact and permeabilized platelets, and implicate 1,2-diacylglycerol as the factor responsible for the enhancement by thrombin of ^{14}C -serotonin secretion²⁵. Production of 1,2-diacylglycerol on stimulation by thrombin has been observed in both intact⁹ and permeabilized (R. J. Haslam, personal communication) platelets, although in the latter preparation the expected phosphoinositide breakdown²⁶ has not yet been demonstrated²⁷.

Addition of 20 μM cyclic AMP or cyclic GMP has no significant effect on the dose-response curve for ^{14}C -serotonin secretion induced by addition of Ca^{2+} when added alone (Fig. 2b). However, in the presence of thrombin (Fig. 2a), addition of 20 μM cyclic AMP depresses the response to a level approaching that observed in the presence of Ca^{2+} alone by shifting the dose-response curve to the right, whereas addition of 20 μM cyclic GMP enhances the response to Ca^{2+} by shifting the dose-response curve to the left (Fig. 2a). Dose-response curves obtained for the effects of cyclic AMP and cyclic GMP determined in the presence of thrombin and at four fixed calculated Ca^{2+} concentrations (Fig. 3) provide further evidence for specificity. Neither cyclic nucleotide has any significant effect at very low or saturating Ca^{2+} concentration where addition of thrombin causes little, if any, increment in ^{14}C -serotonin secretion (Figs 1, 3). At intermediate Ca^{2+} concentrations the presence of cyclic AMP markedly inhibits ^{14}C -serotonin secretion, with half-maximal inhibition obtained at or below 10^{-6} M (Fig. 3) whereas addition of cyclic GMP enhances ^{14}C -serotonin secretion with an EC_{50} in the micromolar range (Fig. 3). This effect of cyclic-3'5'-GMP is not mimicked by addition of cyclic-2'3'-GMP (Fig. 3 legend).

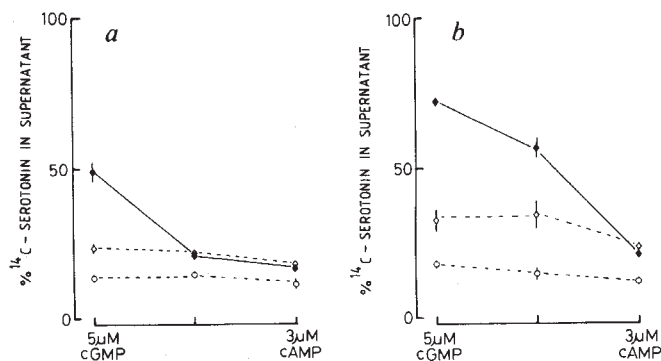


Fig. 4 Effect of raising the OAG and thrombin concentrations at fixed calculated Ca^{2+} concentrations of 0.17 μM (a) and 0.4 μM (b) in the presence and absence of cyclic AMP and cyclic GMP. Platelets were prepared and rendered permeable as described for Fig. 2. After incubation for 25 min at 22 °C the permeabilized platelet preparations ($\sim 3 \times 10^8$ cells ml^{-1}) were incubated for a further 5 min at 22 °C with 20 mM Ca-EGTA buffers corresponding to 0.17 μM Ca^{2+} (a) and 0.4 μM Ca^{2+} (b). 1.2-ml aliquots of the cell suspensions were then incubated for a further 5 min at 22 °C with 0.2 μM IBMX in the absence or presence of 3 μM cyclic AMP or 5 μM cyclic GMP. 0.1-ml aliquots of these suspensions were then added to 0.05 ml of a cell suspension medium containing the same Ca-EGTA buffers, in the absence of other additions (○) or in the presence of OAG (◇) or thrombin (◆). The final concentrations of thrombin and OAG were 0.6 $U\ ml^{-1}$ and 7.5 $\mu g\ ml^{-1}$, respectively. The cell suspensions were incubated for 5 min at 22 °C before being centrifuged, and the supernatant fraction was assayed for ^{14}C content as described for Fig. 1. Data points refer to the mean of four determinations, with the error bars showing the s.e.m. The difference in the relative extents of the responses to thrombin and OAG as compared with their similarity in Fig. 1 results from the use of different OAG concentrations in these two experiments.

Studies similar to those shown in Fig. 2 have been performed using OAG instead of thrombin. Addition of cyclic AMP (20 μM) reduces the extent to which OAG shifts the dose-response curve for activation by Ca^{2+} to the left. However, the effect of cyclic AMP on the Ca^{2+} -dependent response to OAG or TPA occurs at a much higher concentration ($IC_{50} > 10\ \mu M$) than that which inhibits the response to thrombin ($IC_{50} = 0.3\ \mu M$) (data not shown but see Fig. 4). The effect of these cyclic nucleotides on the secretory response to thrombin or OAG has also been examined in conditions which partially simulate conditions in the intact cell. At a Ca^{2+} concentration of 0.17 μM , which approaches that typical of unstimulated intact platelets⁵, addition of thrombin or OAG causes little increase in the secretory response. This small response is not obviously inhibited by 3 μM cyclic AMP. The response to thrombin, but not to OAG, is enhanced by addition of 5 μM cyclic GMP (Fig.

4a), in accord with the ability of cyclic GMP to enhance only the increase in the Ca^{2+} sensitivity of thrombin-induced secretion (Fig. 2 and data not shown). At a higher Ca^{2+} concentration (0.4 μM) which is still below the threshold for induction of shape change by ionomycin⁵, a significant secretory response results from addition of either thrombin or OAG (Fig. 4b). At this higher Ca^{2+} concentration the response to thrombin is further enhanced in the presence of 5 μM cyclic GMP but is clearly inhibited in the presence of 3 μM cyclic AMP. The response to OAG is, in contrast, unaffected by the presence of cyclic GMP and is only slightly reduced by cyclic AMP (Fig. 4b).

These data therefore suggest that cyclic nucleotide control is primarily, although not exclusively, exerted before the formation of 1,2-diacylglycerol. Our data provide strong support for inhibition of phospholipase C by cyclic AMP, which has previously been proposed on the basis of data obtained using dibutylcyclic AMP and intact platelets^{15,16}. This inhibitory effect may be exerted directly on phospholipase C or on a factor which regulates the supply of substrate to this enzyme. However, another inhibitory site for cyclic AMP lies beyond phospholipase C, on the stimulus-response coupling pathway, but becomes important only at higher cyclic AMP concentrations. The effect of cyclic GMP is, in contrast, restricted to enhancement of the secretory response induced by thrombin at submaximal levels of Ca^{2+} (Figs 2–4), and may therefore result wholly from direct or indirect activation of phospholipase C. Such an effect is inconsistent with results obtained using intact platelets in which an increase in [cyclic GMP]_i has been reported to inhibit secretion and 1,2-diacylglycerol formation^{8,28,29} but accords with a study in which addition of dibutylcyclic GMP enhanced platelet aggregation and secretion¹². The reason for the discrepancy with the former studies is unclear, although the pH and temperature used here differ substantially from the conditions in which the response of the intact cell is usually measured. However, the intact cell data may be equivocal as certain agents, for example, sodium nitroprusside, which increase [cyclic GMP]_i, also less effectively raise [cyclic AMP]_i (ref. 28), while others, such as ascorbic acid, fail to inhibit the aggregatory response³⁰. Furthermore, inhibition of responses by 8-bromo-cyclic GMP^{8,28,29} is not definitive because in other systems the effects of membrane-permeant cyclic nucleotide derivatives on intact cells have not always been consistent with results obtained by direct introduction of cyclic nucleotides into the cytosol³¹.

Finally, if inositol-1,4,5-trisphosphate, another product of the breakdown of triphosphoinositide by phospholipase C, causes mobilization of Ca^{2+} to the cytosol as described for the pancreatic acinar cell^{32,33}, control of this enzyme by cyclic nucleotides could provide coordinate regulation of Ca^{2+} mobilization and 1,2-diacylglycerol formation in the platelet.

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- Rasmussen, H. *Calcium and cAMP as Synaptic Messengers* (Wiley, New York, 1981).
- Mills, D. C. B. & Smith, J. B. *Biochem. J.* **121**, 185–196 (1971).
- Steer, M. & Salzman, E. *Adv. Cyclic Nucleotide Res.* **12**, 71–94 (1980).
- Kaibuchi, K., Sano, K., Hoshijima, M., Takai, Y. & Nishizuka, Y. *Cell Calcium* **3**, 323–335 (1982).
- Rink, T. J., Smith, S. W. & Tsien, R. Y. *FEBS Lett.* **148**, 21–26 (1982).
- Hallam, T. J., Rink, T. J. & Sanchez, A. *J. Physiol., Lond.* **343**, 98P (1983).
- Rink, T. J., Sanchez, A. & Hallam, T. J. *Nature* **305**, 317–319 (1983).
- Nishizuka, Y. *Phil. Trans. R. Soc. B302*, 101–112 (1983).
- Rittenhouse-Simmons, S. & Deykin, D. in *Platelets in Biology and Pathology* Vol. 2 (ed. Gordon, J. L.) 349–372 (Elsevier, Amsterdam, 1981).
- Mills, D. C. B. *CIBA Fdn Symp.* **35**, (New ser.) 153–167 (1975).
- Gorman, R. R., Fitzpatrick, F. A. & Miller, O. V. *Biochem. biophys. Res. Commun.* **79**, 305–313 (1977).
- Chiang, T. M., Dixit, S. N. & Kang, A. H. *J. Lab. clin. Med.* **88**, 215–221 (1976).
- Rink, T. J. & Smith, S. W. *J. Physiol., Lond.* **338**, 66P–67P (1983).
- Kazer-Glanzmann, R., Jakabova, M., George, J. N. & Luscher, E. F. *Biochim. biophys. Acta* **446**, 429–440 (1977).
- Rittenhouse-Simmons, S. *J. clin. Invest.* **63**, 580–587 (1979).
- Billah, M. M., Lapetina, E. G. & Cuatrecasas, P. *Biochem. biophys. Res. Commun.* **90**, 92–98 (1979).
- Baker, P. F. & Knight, D. E. *Nature* **276**, 620–622 (1978).

- Knight, D. E. in *Techniques in Cellular Physiology* Vol. 1 (ed. Baker, P. F.) 113, 1–20 (Elsevier, Amsterdam, 1981).
- Knight, D. E. & Scrutton, M. C. *Thromb. Res.* **20**, 437–446 (1980).
- Mori, T. et al. *J. Biochem., Tokyo* **91**, 427–431 (1982).
- Castagna, M. et al. *J. Biol. Chem.* **257**, 7847–7851 (1982).
- Yamanishi, J. et al. *Biochem. biophys. Res. Commun.* **112**, 778–786 (1983).
- Knight, D. E. & Baker, P. F. *FEBS Lett.* **160**, 98–100 (1983).
- Knight, D. E., Hallam, T. J. & Scrutton, M. C. *Nature* **296**, 256–257 (1982).
- Knight, D. E. & Scrutton, M. C. *Thromb. Haemostas.* **50**, 93 (1983).
- Michell, R. H., Kirk, C. J., Jones, L. M., Downes, C. P. & Creba, J. A. *Phil. Trans. R. Soc. B296*, 123–137 (1981).
- Hallam, T. J. thesis, Univ. London (1983).
- Haslam, R. J., Salama, S. E., Fox, J. E. B. & Davidson, M. M. L. in *Platelets: Cellular Response Mechanisms and their Biological Significance* (eds Rotman, A., Meyer, F. A., Gilter, C. & Silberberg, A.) 213–231 (Wiley, New York, 1980).
- Takai, Y., Kaibuchi, K., Matsubara, T. & Nishizuka, Y. *Biochem. biophys. Res. Commun.* **101**, 61–67 (1981).
- Weiss, A., Baenziger, N. L. & Atkinson, J. P. *Blood* **52**, 524–531 (1978).
- Knight, D. E. & Koh, E. *J. Physiol., Lond.* **345**, 126P (1983).
- Berridge, M. J. *Biochem. J.* **212**, 849–858 (1983).
- Streb, H., Irvine, R. F., Berridge, M. J. & Schulz, I. *Nature* **306**, 67–69 (1983).
- Martell, A. & Sillen, L. G. *Chem. Soc. Spec. Publ.* **17**, 697–698 (1964).
- Tsien, R. Y. *Biochemistry* **19**, 2396–2404 (1980).

Dihydropyridine BAY-K-8644 activates chromaffin cell calcium channels

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Douglas and Rubin¹ suggested that "the role of acetylcholine as a transmitter at the adrenal medulla is to cause some brief change in medullary cells which allows Ca ions to penetrate them and trigger the catecholamine ejection process". The Ca²⁺-channel blocking agents, verapamil, nifedipine and nitrendipine, have been used widely to investigate the properties of slow Ca²⁺ channels in a variety of tissues², including the adrenomedullary chromaffin cell^{3,4}. Recently, small modifications to the nifedipine molecule produced a derivative, BAY-K-8644 (methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate), that in contrast to the Ca²⁺-channel blocking agents, stimulated cardiac and vascular smooth muscle contractility⁵. We have tested whether this compound behaves as a Ca²⁺-channel activator at the chromaffin cell membrane as shown by Schramm *et al.*⁵ in smooth muscle cells. The experiments described here strongly suggest that it does so.

Cat adrenal glands were isolated and prepared for retrograde perfusion with Krebs-bicarbonate solution at room temperature⁶. After 1 h initial perfusion with oxygenated Krebs-bicarbonate solution, spontaneous catecholamine outputs from cat adrenal glands amounted to 187 ± 24 ng per 10 min ($N = 75$). When the glands were subsequently stimulated for 2 min, at 30-min intervals, with isotonic solutions containing increasing concentrations of K⁺, the net release of catecholamines was directly proportional to the concentration of K⁺ used up to 59 mM. BAY-K-8644 at concentrations of 10^{-8} – 10^{-4} M did not affect spontaneous catecholamine output; in the presence of BAY-K-8644 (1 μ M), the release was 145 ± 28 ng per 10 min ($N = 42$). A drug concentration of 1 μ M markedly increased catecholamine release evoked by 17.7 mM K⁺; while evoked net release by K⁺ was 2.93 ± 0.38 μ g per 8 min ($N = 27$), in the presence of 1 μ M BAY-K-8644 it was 10.1 ± 1.51 μ g per 8 min ($N = 14$). Secretory responses to 59 mM K⁺ were not affected by the drug. These results suggest that BAY-K-8644 is not acting on quiescent chromaffin cells and that a moderate depolarizing stimulus is required for the drug to manifest its potentiating effects on secretion. If this agent were acting on Ca²⁺ channels, the lack of potentiation of the secretory responses to higher K⁺ concentrations might indicate that in these conditions, all channels are in their maximal state of activation or their most inactivated form.

Since the potentiation by BAY-K-8644 was greater at moderately depolarizing stimuli, 17.7 mM K⁺ solutions were used to test the effects of varying concentrations of the drug on K⁺-evoked catecholamine release. Six 2-min pulses of K⁺ were given to perfused cat adrenal glands at 20-min intervals; moderate catecholamine release rates were obtained. The release was slowly decaying from the first to the sixth pulse, the amount of catecholamines found in S₆ being about 50% of that obtained in S₁ (Fig. 1a). In the contralateral paired gland, BAY-K-8644 started to potentiate catecholamine release at 10^{-8} M, and had already reached maximum potentiation at 10^{-7} M (Fig. 1a); at 10^{-6} and 10^{-5} M the potentiating effect was similar, but at 10^{-4} M the secretory response to K⁺ fell abruptly, probably because at these high concentrations the drug behaves as a Ca²⁺-channel blocking agent. The great potentiation obtained with low concentrations of BAY-K-8644 suggests a highly selec-

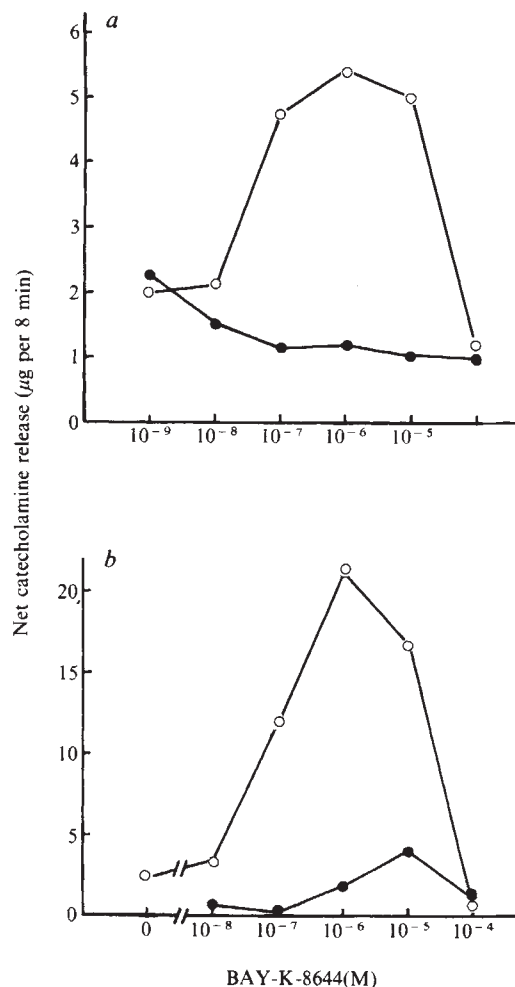


Fig. 1 Effects of increasing concentrations of BAY-K-8644 on the net release of catecholamines evoked by repeated 2-min pulses of 17.7 mM K⁺ (NaCl substituted by equiosmolar amounts of KCl), given to perfused cat adrenal glands at 30-min intervals. *a*, Six pulses of K⁺ were given in the absence or presence of increasing concentrations (abscissa) of BAY-K-8644. These are the results of a paired typical experiment (out of four) in which one gland was free of drug (●) and the contralateral gland was perfused with 10^{-9} – 10^{-4} M BAY-K-8644 in a cumulative manner (○); each concentration of drug was added 10 min before, during the K⁺ pulse and until the following concentration was introduced. *b*, A paired experiment; one gland was perfused with BAY-K-8644 at the concentrations shown on the abscissa (○); the contralateral gland was perfused in addition to this drug, with the Ca²⁺ channel antagonist nitrendipine (10^{-6} M present 10 min before the first K⁺ pulse and during the rest of the experiment) (●). Total catecholamine content of perfusate samples (noradrenaline plus adrenaline)⁸. Net catecholamine release during high K⁺ was calculated by subtracting the basal, spontaneous release from the release obtained in the 2-min high-K⁺ sample plus that obtained in three additional 2-min samples and expressed as μg per 8 min.

tive effect; since high K⁺ is presumed to activate catecholamine secretion by directly depolarizing the chromaffin cell and opening voltage-sensitive Ca²⁺ channels^{3,7,8}, it seems feasible to assume that the target receptor for the drug is the slow Ca²⁺ channel. The fact that nitrendipine (a Ca²⁺-channel blocking agent that potently inhibits catecholamine release evoked by nicotine or high K⁺)³ antagonizes the potentiating effect of BAY-K-8644 on K⁺-evoked secretion (Fig. 1b), is consistent with this conjecture.

If BAY-K-8644 activates Ca²⁺ channels, it should favour the influx of ⁴⁵Ca by the chromaffin cell; this was certainly the case, as shown in the experiments of Fig. 2. The uptake of ⁴⁵Ca by acutely isolated chromaffin cells suspended in oxygenated Krebs-bicarbonate solution was substantially increased in the

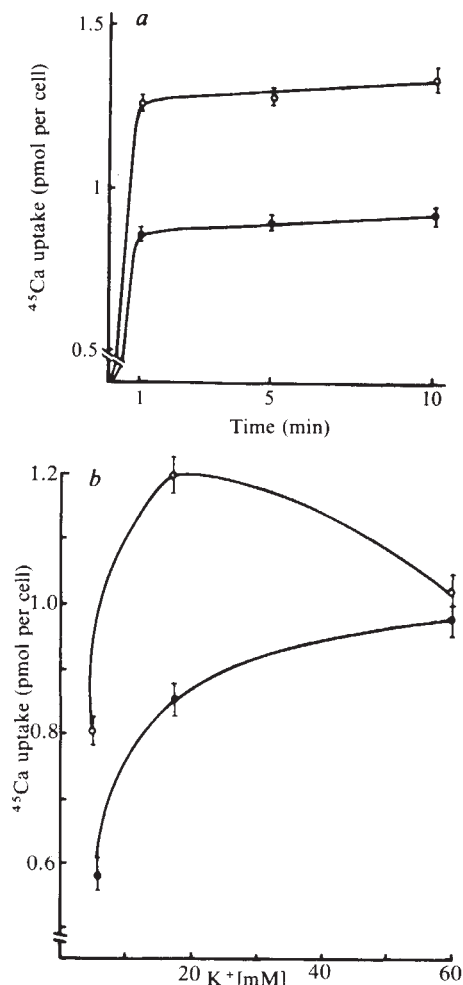


Fig. 2 BAY-K-8644 increases ^{45}Ca uptake into acutely isolated bovine adrenal chromaffin cells prepared as previously described¹⁹ (○, BAY-K-8644; ●, control). **a**, Time course of the uptake of ^{45}Ca by chromaffin cells exposed to high K^+ (17.7 mM) solution in the absence (control) or the presence of BAY-K-8644 (1 μM). Immediately after isolation and washing, cells were incubated in oxygenated Krebs-bicarbonate solution at 37 °C for 15 min; then aliquots of the cell suspension containing 5×10^5 cells were added to 1 ml Krebs solution with 17.7 mM K^+ (NaCl reduced equiosmotically) and 3×10^6 c.p.m. ^{45}Ca per assay (Amersham; specific activity 20.1 mCi mg^{-1}). In BAY-K-8644-treated cells, the drug (1 μM) was present 15 min before adding K^+ and ^{45}Ca . One, five or ten minutes after adding ^{45}Ca and K^+ , 3 ml Krebs solution (with or without BAY-K-8644) were added and the cells centrifuged at 2,000 r.p.m. for 10 min; cells were resuspended and washed four times in the above solution. Finally, they were vigorously resuspended in 2 ml of 0.4 M perchloric acid and centrifuged at 2,000 r.p.m. for 10 min; 0.5 ml of the supernatants were added to 5 ml Instagel (Packard) and the radioactivity was counted in a LS2800 Beckman scintillation counter. ^{45}Ca taken up and retained by cells was expressed as pmol per cell (ordinate). Data are means $\pm$ s.e. of eight experiments. **b**, Effects of BAY-K-8644 on ^{45}Ca uptake by chromaffin cells suspended in solutions containing 5.9 (normal Krebs), 17.7 or 59 mM K^+ . BAY-K-8644 (1 μM) was present 15 min before and during K^+ stimulation. Conditions were as above. Results are means $\pm$ s.e. of eight experiments.

presence of 17.7 mM K^+ , in relation to the uptake observed in non-stimulated cells; uptake was almost complete after 1 min incubation, although it continued to rise slightly after 5 or 10 min incubation (Fig. 2a). When cells were suspended in the presence of BAY-K-8644 at a concentration (10^{-6} M) which maximally potentiated K^+ -evoked catecholamine release (Fig. 1a), ^{45}Ca uptake-retention in the presence of 17.7 mM K^+ was enhanced at all incubation times studied (Fig. 2a). Figure 2b shows the uptake of ^{45}Ca in the presence of 17.7 or 59 mM K^+ ; BAY-K-8644 increased ^{45}Ca influx significantly ($P < 0.001$, $N = 8$) at

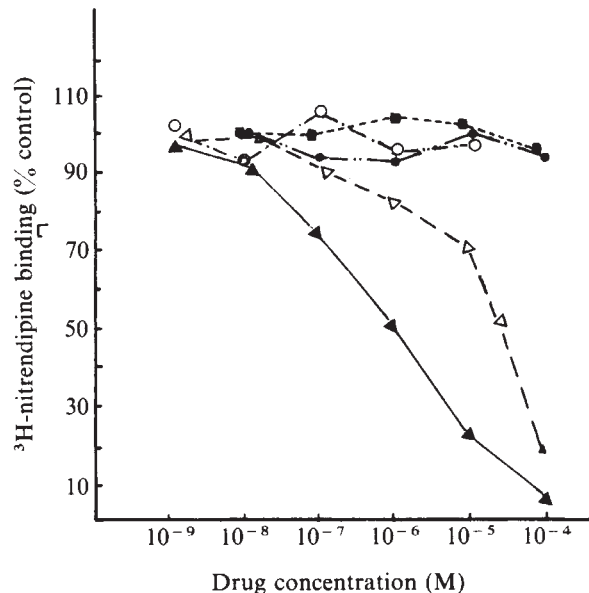


Fig. 3 Specific binding of ^3H -nitrendipine to bovine adrenomedullary membrane fragments and its displacement by BAY-K-8644. A crude microsomal fraction isolated from bovine adrenal medulla homogenates (0.3 M sucrose containing 10 mM Tris buffer, pH 7.4) was used as the source of membranes. The reaction mixture consisted of 300 μg membrane protein, 50 mM Tris buffer pH 7.4, 1 nM ^3H -nitrendipine and increasing concentrations of displacing agent (abscissa). Incubation was at 37 °C under a sodium lamp for 30 min. The ordinate shows ^3H -nitrendipine specifically bound to membranes as % of maximal binding in the absence of displacing agent. IC_{50} values for nitrendipine and BAY-K-8644 were, respectively, 1 and 50 nM. Data are means $\pm$ s.e. of three to six experiments. ○, Imipramine; ●, cocaine; ■, nicotine; △, BAY-K-8644; ▲, nitrendipine.

the lower K^+ concentration but not when 59 mM K^+ was used, suggesting once more that at higher K^+ concentrations, Ca^{2+} channels might be fully activated and that ^{45}Ca entry through them cannot be further increased. Note that BAY-K-8644 also slightly but significantly enhanced the ^{45}Ca taken up by quiescent cells ($P < 0.01$, $N = 8$); this finding contrasts with the lack of effect of the drug on spontaneous catecholamine release or rabbit aorta contractility⁵. Even so, these experiments support the suggestion that this agent is promoting Ca^{2+} uptake through specific voltage-sensitive Ca^{2+} channels.

^3H -nitrendipine has been used as a specific radioligand to characterize the dihydropyridine receptor probably associated with the Ca^{2+} channel in various tissues⁹ including the adrenal medulla¹⁰. Since nitrendipine antagonized the potentiating effects of BAY-K-8644 on catecholamine release evoked by high K^+ , the possible modification of ^3H -nitrendipine binding to membranes could help to define further its presumed binding site on the chromaffin cell membrane and potentiation of K^+ -evoked catecholamine release. Measurements of ^3H -nitrendipine-specific binding to membrane fragments from bovine adrenal medulla demonstrate a saturable binding with an apparent K_D of 1.18 ± 0.32 nM and a B_{max} of 325.4 ± 136 fmol per mg protein ($N = 4$). These values are compatible with the presence in the chromaffin cell membrane of a high-affinity binding site probably associated with the voltage-dependent Ca^{2+} channel. The specificity of the radioligand for the Ca^{2+} channel is further corroborated by the fact that agents which bind to the acetylcholine site (nicotine) or to the ionophoric site (imipramine, cocaine)³ of the nicotinic cholinergic receptor did not affect ^3H -nitrendipine binding (Fig. 3). In contrast, BAY-K-8644 fully displaced ^3H -nitrendipine bound to membranes, suggesting once more that this drug is acting on the voltage-sensitive Ca^{2+} channel.

Studies involving measurements of catecholamine release

evoked by depolarizing agents^{3,11-13}, and monitoring of ⁴⁵Ca transmembrane fluxes¹⁴⁻¹⁶ or electrical recordings using patch-clamp techniques¹⁷ have indicated the presence in the chromaffin cell membrane of slow Ca²⁺ channels which are sensitive to voltage changes. The potency of nitrendipine in inhibiting³ and that of BAY-K-8644 in enhancing K⁺-evoked catecholamine release suggests that the chromaffin cell membrane might contain a 'dihydropyridine' receptor closely related to this voltage-sensitive slow Ca²⁺ channel; if so, it is likely that an endogenous modulator for such a receptor might be manufactured by the cell to control the activation and inactivation of Ca²⁺ channels either from the cytosol or from the cell exterior after its

exocytotic release, as suggested for smooth muscle cells⁵. In any case, the dihydropyridine BAY-K-8644 might be the first compound available to manipulate pharmacologically the rates of activation or inactivation of Ca²⁺ channels; if so, it will prove a useful tool for studying the properties of such channels as well as their functional dynamics during the secretory cycle for catecholamines and other neurotransmitters and hormones.

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1. Douglas, W. W. & Rubin, R. P. *J. Physiol., Lond.* **159**, 40-57 (1961).
2. Fleckenstein, A. A. *Rev. Pharmac. Tox.* **17**, 149-166 (1977).
3. Ceña, V., Nicolas, G. P., Sánchez-García, P., Kirpekar, S. M. & García, A. G. *Neuroscience* (in the press).
4. Pinto, J. E. B. & Trifaró, J. M. *Br. J. Pharmac.* **57**, 127-132 (1976).
5. Schramm, M., Thomas, G., Towart, R. & Franckowiak, G. *Nature* **303**, 535-537 (1983).
6. García, A. G., Hernández, M., Horga, J. F. & Sánchez-García, P. *Br. J. Pharmac.* **68**, 571-583 (1980).
7. Douglas, W. W., Kanno, T. & Sampson, S. R. *J. Physiol., Lond.* **191**, 107-121 (1967).
8. Ishikawa, K. & Kanno, T. *Jap. J. Physiol.* **28**, 275-289 (1978).
9. Bellemann, P., Ferry, D., Lübbecke, F. & Glossmann, H. *Arzneim. Forsch.* **31**, 2064-2067 (1981).

10. Frías, J., Alonso, F. G., Ceña, V., Sánchez-García, P. & García, A. G. in *Molecular Neurobiology of Peripheral Catecholaminergic Systems* (ed. García, A. G.) 151 (Universidad Autónoma de Madrid, Madrid, 1982).
11. Baker, P. F. & Rink, T. J. *J. Physiol., Lond.* **253**, 593-620 (1975).
12. Kirpekar, S. M. & Prat, J. C. *Proc. natn. Acad. Sci. U.S.A.* **76**, 2081-2083 (1979).
13. Kirpekar, S. M., García, A. G. & Schiavone, M. T. *Adv. Biosci.* **36**, 55-62 (1982).
14. Douglas, W. W. & Poisner, A. M. *J. Physiol., Lond.* **162**, 385-392 (1962).
15. Kilpatrick, D. L., Slepets, R. L., Corcoran, J. J. & Kirshner, N. *J. Neurochem.* **38**, 427-435 (1982).
16. Holz, R. W., Senter, R. A. & Frye, R. A. *J. Neurochem.* **39**, 635-646 (1982).
17. Fenwick, E. M., Marty, A. & Neher, E. *J. Physiol., Lond.* **331**, 599-635 (1982).
18. Shellenberger, M. K. & Gordon, J. H. *Analyt. Biochem.* **39**, 356-372 (1971).
19. Aunis, D. & García, A. G. *Br. J. Pharmac.* **72**, 31-40 (1981).

A haemoglobin switching activity modulates hereditary persistence of fetal haemoglobin

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During human development there is a switch from fetal to adult haemoglobin formation, reflecting the differential expression of fetal (ϵ and γ) and adult (β and δ) globin genes. Mutations that inhibit this switch produce variants of the syndrome of hereditary persistence of fetal haemoglobin (HPFH). Adult heterozygotes for these mutants produce 15-30% fetal haemoglobin (HbF) in their red cells¹. The general assumption is that the mutations result in a permanent switching on of γ -globin genes. Here, however, we show that fetal globin expression can be turned off in cultures of HPFH cells by an uncharacterized factor in fetal sheep serum. This is the first demonstration that mutations affecting the developmental expression of globin genes can be modulated by exogenous factors. The findings raise the possibility that the phenotype of HPFH is not simply the direct result of mutations in or around globin genes but the consequence of the mutations on the interaction of globin genes with *trans*-acting regulatory factors.

The studies described here followed the observation that an activity (named haemoglobin switching activity²⁻⁴) present in sera from sheep fetuses has profound effects on HbF expression in cultures of human erythroid cells. This activity (the nature of which remains unknown) turns off HbF synthesis in cultures of erythroid progenitors from normal adults, from persons with activated HbF synthesis *in vivo*, and from patients with sickle cell anaemia or a β -thalassaemia syndrome²⁻⁴. The activity induces switching of HbF to HbA synthesis in neonatal erythroid progenitor cultures, but fails to induce HbF to HbA switching in cultures of fetal burst-forming units (BFU-E)²⁻⁴. *In vitro* studies suggest that haemoglobin switching occurs through a direct interaction with early as well as late erythroid progenitors³.

The availability of culture conditions that can modify HbF expression has enabled us to study HPFH mutations. HPFH

mutations with *cis* ϵ and γ but not β and δ ($\epsilon\gamma\gamma$ HPFH) globin expression or *cis* ϵ ($\epsilon\gamma$ HPFH) or γ ($\gamma\gamma$ HPFH) chain synthesis are known¹. Previous structural analyses have revealed various deletions in the β -globin genomic regions of $\epsilon\gamma\gamma$ HPFH mutants but no globin gene abnormalities in the $\epsilon\gamma$ or $\gamma\gamma$ HPFH mutants⁵⁻¹⁵. How these mutations continue to express HbF in the adult and whether the structural anomalies of the $\epsilon\gamma\gamma$ HPFH mutations affect the normal mechanism that controls haemoglobin switching, remain unknown.

In the present study, erythroid progenitors (BFU-E) were cultured in the conditions described in Fig. 1 legend. Cells were grown in semisolid media and mature erythroid clones collected after 15-17 days in culture, incubated with ³H-leucine and used to measure the incorporation of radioactivity into the globin chains as described previously^{16,17}. Media in control cultures contained fetal calf serum (FCS). In the experimental cultures, FCS was substituted by a preparation of fetal sheep serum (FSS) which potentially induced HbF to HbA switching in cultures of neonatal BFU-E and BFU-E from persons with an acquired HbF elevation.

As shown in Fig. 1, cultures of cells from two $\epsilon\gamma\gamma$ HPFH heterozygotes (having a mutation that deletes part of the ϵ to δ flanking region, the δ and β genes and several kilobases 3' of the β gene) produced 25-40% HbF in the control FCS cultures. In FSS cultures, however, HbF synthesis ranged from barely detectable to a maximum of 2%. Cells from two other $\epsilon\gamma\gamma$ HPFH carriers, in whom the molecular lesion has not been defined, showed a similar degree of abrogation of HbF synthesis in FSS cultures. To test the response of non-deletion HPFH variants, we cultured erythroid cells from a heterozygote for the Greek γ HPFH and a heterozygote for the African γ HPFH. These cells produced 20-35% HbF in FCS cultures, but barely measurable amounts in FSS cultures (Fig. 1).

Several experiments were done to determine the reason for the inhibition of HPFH expression in FSS cultures. It is known that γ and β globins are produced asynchronously during erythroblast maturation, γ globin and γ mRNA accumulating in immature erythroblasts, with β -globin expression increasing in mature erythroblasts^{18,19}. To test whether FSS decreased HbF through this maturational mechanism, deletion HPFH BFU-E were cultured and immature clones (composed of proerythroblasts and basophilic erythroblasts) or mature clones (composed of polychromatophilic and orthochromatic erythroblasts) were collected separately and used to assess globin biosynthesis. We found that the immature HPFH erythroblasts had the low γ -globin synthesis characteristic of FSS cultures, a result eliminating the possibility of maturational modulation and suggesting

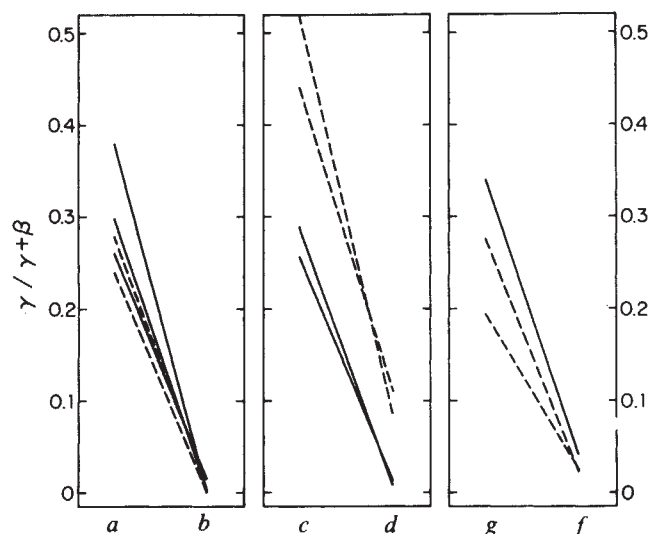


Fig. 1 Globin chain biosynthesis (expressed as $\gamma/\gamma+\beta$ ratio) in cultures of erythroid cells from humans heterozygous for hereditary persistence of fetal haemoglobin. *a*, Cultures performed in FCS. *b*, Cultures performed with FSS containing haemoglobin switching activity. Each line represents the results of a separate experiment. Left panel: results with cells of two $G_{\gamma}^A_{\gamma}$ HPFH heterozygotes who, by globin gene restriction endonuclease mapping, were found to carry the Ghana type deletion HPFH mutation^{11,15}. Middle panel: results from cultures of two $G_{\gamma}^A_{\gamma}$ HPFH heterozygotes whose mutation has not been molecularly characterized. Right panel: findings in two non-deletion mutations, $G_{\gamma}^A_{\gamma}$ HPFH (—) and Greek A_{γ} HPFH (---). Note in all panels the striking reduction of γ -globin synthesis in *b*. In addition to the striking differences in $\gamma/\gamma+\beta$ ratios, the α /non- α ratios differed between FCS and FSS cultures (being 10–20% higher in the latter). This is expected, as turning off the γ -chain, synthesis in the mutant HPFH chromosome will result in α /non- α chain imbalance.

Methods: Peripheral blood was used as a source of erythroid progenitors. After Hypaque-Ficoll centrifugation interphase cells were collected, washed and adhered to plastic surfaces to remove the majority of adherent cells. Non-adherent cells were inoculated at appropriate concentrations ($1-3 \times 10^5 \text{ ml}^{-1}$) in methylcellulose in the presence of erythropoietin, T-cell-derived conditioned medium and either FCS or a FSS preparation known to contain Hb switching activity. In these conditions erythroid progenitors (burst forming units, BFU-E) produce large, multi-lobed erythroid colonies (erythroid bursts) that mature in culture during days 13–20. Mature erythroid bursts were lifted from the plates, pooled according to experimental category and used to measure globin chain synthesis.

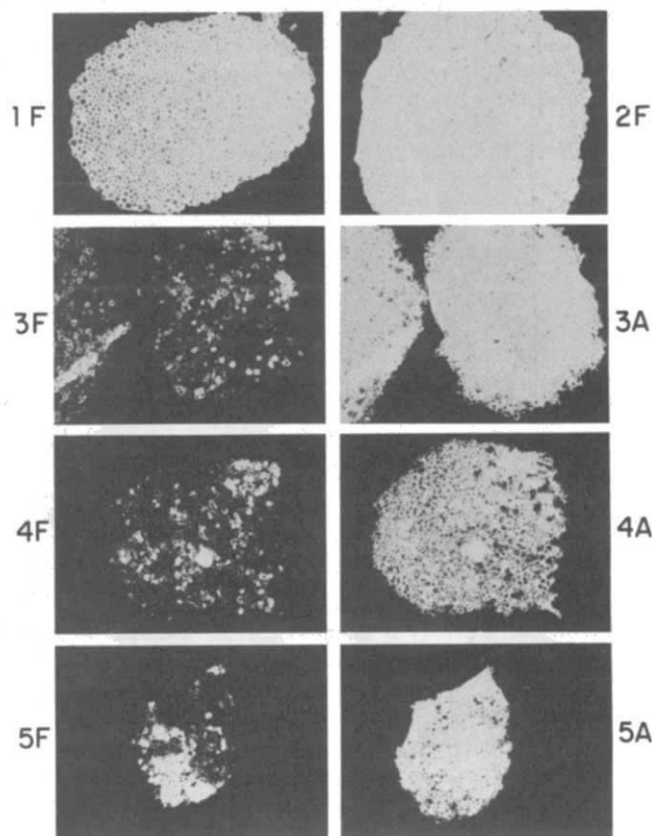


Fig. 2 Immunofluorescent labelling of HPFH clones. Mature clones were lifted from the cultures, fixed and labelled with an anti- γ chain monoclonal antibody and rabbit anti-mouse $F(ab)_2$ -fluorescein isothiocyanate (FITC) and counter-labelled with an anti- β globin chain monoclonal antibody conjugated to rhodamine. This double-labelling approach allows detection of both γ and β globins in each cell of a clone. 1F and 2F show two FCS-grown clones labelled with the anti- γ globin-FITC. Note the strong labelling of all the cells. 3, 4 and 5 are FSS-grown clones labelled with anti- γ FITC (3F, 4F, 5F) and anti- β rhodamine (3A, 4A, 5A). Note that in 3F, 4F and 5F the majority of cells either fail to be labelled by the anti- γ -chain antibody or are labelled very faintly, showing the reduced total accumulation of HbF in the cells of FSS-grown clones.

that the FSS-grown immature HPFH erythroblasts already have been programmed to produce low amounts of HbF. To test whether our findings reflected an absolute reduction of γ -globin chain production, we performed experiments using fluorescent anti-globin chain monoclonal antibodies. A double-labelling procedure (described in Fig. 2 legend) was used and over 50 clones (per experiment) were analysed for γ - and β -globin expression. Clones grown in FCS displayed the pancellular expression of HbF characteristic of HPFH mutations (Fig. 2) whereas in most FSS-grown HPFH clones, γ globin was barely detectable. In other experiments, some cells from FSS-grown clones expressed HbF (Fig. 2). These results showed that culture in FSS results in a marked reduction of γ -chain expression in the HPFH erythroblasts.

To test directly whether HbF was turned off through an interaction between HPFH cells and the switching activity in FSS cultures, experiments were performed using split clones. Deletion HPFH BFU-E were cultured in either FCS or FSS. When the clones had reached a size of about 50 cells (five to six progenitor cell divisions), each clone was divided into two almost equal parts, one part being transferred to a plate containing FCS, the other part to a plate containing FSS. The clones were left to grow and mature (five to six cell divisions) and then

used for globin analysis. As shown in Table 1, there are striking differences in HbF production between the parts of a clone transferred to FSS or to FCS, showing directly that the progeny of the same HPFH progenitor can produce high or low HbF depending on the environment. Of special significance are the low HbF levels observed in the FSS to FCS transfers (Table 1); they show that the expression of the HPFH mutation can be altered even when the cells forming a clone are exposed to the inductive environment only during the initial stages of their *in vitro* differentiation.

The present findings in HPFH cultures are identical to findings in normal and haemoglobinopathic cell cultures reported by us previously²⁻⁴. The fact that the HPFH cells return to the normal pattern for adult cells (that is, turning off HbF expression) when cultured in FSS suggests that the HPFH mutations can be modulated by exogenous factors. Previously, the phenotypic consequences of HPFH mutations have been interpreted to mean that in the deletion mutations, a *cis*-acting regulatory element which usually turns off the γ genes is deleted, while the non-deletion HPFH are mutations of *cis*-acting elements that regulate the individual G_{γ} or A_{γ} genes. The results presented here are compatible with an alternative postulate: in HPFH, the response of the mutant globin genomic regions to normal

Table 1 Synthesis of fetal haemoglobin in divided HPFH clones

Clone no.	Initial growth in	$\gamma/\gamma + \beta$ in part of clone grown in	
		FCS	FSS
1	FCS	0.26	0.01
2	FCS	0.35	0.03
3	FCS	0.22	0.01
4	FCS	0.33	0.02
5	FCS	0.24	0.03
6	FCS	0.59	0.02
7	FCS	0.33	0.06
8	FSS	0.18	0.02
9	FSS	0.13	0.01
10	FSS	0.12	0.02
11	FSS	0.07	0.01
12	FSS	0.15	0.03
13	FSS	0.19	0.02
14	FSS	0.15	0.01

Clones were grown in FCS or FSS. At the 30–50-cell stage the clone was divided into two parts by *in situ* micromanipulation. Using an ultrathin pipette, one part of the clone was lifted and implanted in a cell-free methylcellulose plate containing FCS; the other part of the clone was lifted and implanted in a cell-free methylcellulose plate containing FSS. The transferred parts of the clones were left to grow and mature. At maturation the two parts were of similar sizes (as shown by surface counting). The mature clones were plucked out of the plates, and each part was incubated with ^3H -leucine separately and used for globin chain isoelectric focusing to measure the $\gamma/\gamma + \beta$ globin chain synthesis ratio.

trans-acting regulatory elements that control haemoglobin switching may be altered, so that higher levels of *trans*-acting regulatory elements are required to turn off the expression of HbF in the mutant adult cells. The differences in the degree of inhibition of HbF noted in the split clone transfers (Table 1) and in the studies of single clones by immunofluorescence support this postulate. The hypothesis can also explain the paradox that HbF can be turned off *in vitro* in mutants that express this haemoglobin *in vivo* in conditions of normal erythropoiesis.

Previous work has shown that adult erythroid progenitors have an inherent potential to form progeny which express fetal haemoglobin²⁰. This HbF potential is presumably lost during downstream differentiation through interactions between progenitors and the inductive environment of adult haematopoiesis^{2,3,20}. In HPFH mutations, the structural anomalies in the globin genomic region are expected to augment the HbF potential of erythroid progenitors. In the conditions of haematopoietic differentiation that prevail in the marrow of HPFH heterozygotes, the normal level of the inductive environment is not enough to modulate the augmented HbF potential of HPFH progenitors, and terminal cells expressing HbF are formed. In FSS cultures, haemoglobin switching activity is present in excess throughout erythroid cell development, allowing a degree of cell-environment interaction that can turn off the augmented HbF potential of HPFH progenitors; as a result, erythroblasts lacking HbF are produced.

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- Weatherall, D. J. & Glegg, J. B. *The Thalassemia Syndrome* 3rd edn (Blackwell, Oxford, 1981).
- Papayannopoulou, Th., Kurachi, S., Nakamoto, B., Zanjan, E. D. & Stamatoyannopoulos, G. *Proc. natn. Acad. Sci. U.S.A.* **79**, 6579–6583 (1982).
- Stamatoyannopoulos, G., Nakamoto, B., Kurachi, S. & Papayannopoulou, Th. *Proc. natn. Acad. Sci. U.S.A.* **80**, 5650–5654 (1983).
- Stamatoyannopoulos, G., Papayannopoulou, Th., Nakamoto, B. & Kurachi, S. in *Globin Gene Expression and Hemopoietic Differentiation* (eds Stamatoyannopoulos, G. & Neuhuis, A. W.) 347–363 (Liss, New York, 1983).
- Bernards, R. & Flavell, R. A. *Nucleic Acids Res.* **8**, 1521–1534 (1980).
- Jones, R. W., Old, J. M., Wood, W. G., Clegg, J. B. & Weatherall, D. J. *Br. J. Haemat.* **50**, 415–422 (1982).
- Balsley, J. F., Rappaport, E., Schwartz, E. & Surrey, S. *Blood* **59**, 828–833 (1982).
- Old, J. M., Ayyub, H., Wood, W. G., Clegg, J. B. & Weatherall, D. J. *Science* **215**, 981–982 (1982).
- Farquhar, M. et al. *Am. J. hum. Genet.* **35**, 611–620 (1983).
- Fritsch, E. G., Lawn, R. M. & Maniatis, T. *Nature* **279**, 589–603 (1979).

- Tuan, D., Murnane, M. J., DeRiel, J. K. & Forget, B. G. *Nature* **285**, 335–337 (1980).
- Maniatis, T., Fritsch, E. F., Lauer, J. & Lawn, R. M. *A. Rev. Genet.* **14**, 145–178 (1980).
- Ottolenghi, S. et al. *Proc. natn. Acad. Sci. U.S.A.* **79**, 2347–2351 (1982).
- Jagadeeswaran, P., Tuan, D., Forget, B. G. & Weissman, S. M. *Nature* **296**, 469–470 (1982).
- Spritz, R. A. & Forget, B. G. *Am. J. hum. Genet.* **35**, 333–361 (1983).
- Righetti, P. G. et al. *J. biochem. biophys. Meth.* **1**, 45–46 (1979).
- Papayannopoulou, Th., Kurachi, S., Brice, M., Nakamoto, B. & Stamatoyannopoulos, G. *Blood* **57**, 531–536 (1981).
- Papayannopoulou, Th., Kalmantis, Th. & Stamatoyannopoulos, G. *Proc. natn. Acad. Sci. U.S.A.* **76**, 6420–6424 (1979).
- Farquhar, M. N. et al. *Dev. Biol.* **85**, 403–408 (1981).
- Papayannopoulou, Th., Brice, M. & Stamatoyannopoulos, G. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2923–2927 (1977).

Contribution of human $V_{\kappa\text{II}}$ germ-line genes to light-chain diversity

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The genetic basis of the antibody repertoire—estimated to exceed 10^6 different immunoglobulin molecules—is a major unanswered problem^{1,2}. The number of germ-line V_{κ} genes in the mouse genome is probably several hundred^{3,4} while the corresponding number for three out of four human V_{κ} subgroups ($V_{\kappa\text{I}}$, $V_{\kappa\text{III}}$ and $V_{\kappa\text{IV}}$) is probably altogether only 15–20 (ref. 5). The κII proteins differ significantly in sequence from the other κ -chain proteins⁶. To determine the contribution of $V_{\kappa\text{II}}$ genes to κ -chain diversity, we searched for a human lymphoid cell line which produces a κII chain and report here for the first time the sequence of a $V_{\kappa\text{II}}$ gene. According to blot hybridizations with this V_{κ} gene as a probe, subgroup II contributes about half as many genes to the V_{κ} gene repertoire as are detected by a $V_{\kappa\text{I}}$ probe. Therefore the repertoire is rather small which implies that somatic mutations^{7–9} or other mechanisms must play an important role in the generation of light-chain diversity in humans.

Subgroups I–III (refs 10, 11), and IV (ref. 12) were defined on the basis of the amino acid sequences of κ -type Bence-Jones and myeloma proteins. All human κ chains now known fit into this classification⁶. Recently, it has become clear that both a number of germ-line V_{κ} genes and somatic processes, including somatic point mutations, contribute to the sequence variation among light chains^{1,2}. Recent advances in molecular cloning have made it possible to estimate the relative contribution of germ-line V_{κ} genes to the light-chain diversity. $V_{\kappa\text{I}}$ and $V_{\kappa\text{III}}$ probes hybridized not only with each other but also detected, in blot experiments with digests of total human DNA, essentially the same bands corresponding probably to the same 15–20 V_{κ} genes⁵. Since subgroup I and III proteins are more closely related to subgroup IV proteins than to one another, it was suggested⁵ that these 15–20 genes may also contain the $V_{\kappa\text{IV}}$ genes.

The development of subgroup specific antisera¹³ made it possible to assign κ chains to the subgroups without determining the amino acid sequences. Using such antisera in immunodiffusion assays with supernatants from various cell lines we found that the κ chain excreted by the human lymphoblastoid cell line GM 607 belongs to subgroup II. The cell line GM 607 which had been used in a number of studies (see, for example, ref. 14) contains one rearranged and one germ-line C_{κ} gene. The rearranged V_{κ} – C_{κ} region was cloned (Fig. 1) and the V_{κ} gene region sequenced (Fig. 2). The amino acid sequence which was obtained from the DNA sequence confirms the serological assignment of the GM 607 κ chain to subgroup II. Codons 97–108 are derived from the gene segment J1. The assignment of the sequence to subgroup II is unequivocal since all subgroup specific amino acids were found in the characteristic positions⁶ and there is a 98% homology to the published sequence of the

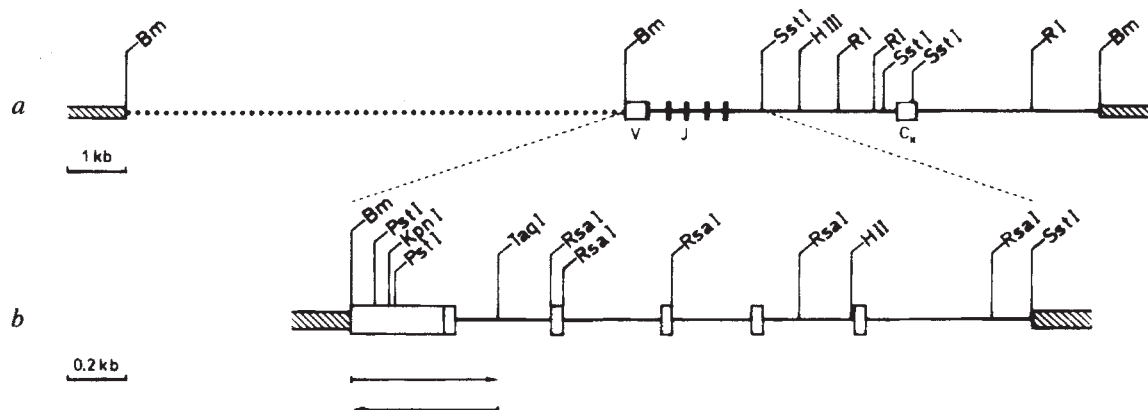


Fig. 1 Restriction map and sequencing strategy of a V_{κ} gene cloned from DNA of the lymphoblastoid cell line GM 607. The cell line which had been derived from a clinically unaffected person was obtained from the Human Genetic Mutant Cell Repository (Camden, New Jersey). A *Bam*HI digest of GM 607 DNA was electrophoretically separated and the 8-kb region cloned in Charon 30 (ref. 20). This is the region which hybridized with a cloned 2.6-kb *Eco*RI fragment from the human C_{κ} region²¹. The C_{κ} clone was derived from a 10.6-kb *Bam*HI clone which in turn had been isolated from human placenta DNA using a mouse J_{κ} region clone (1.56-kb *Hind*III–*Hind*II; ref. 22). From the GM 607 clone (a) which contains an unrelated DNA fragment (.....) in addition to the κ gene, the subclone (b) was prepared in a pBR322 derivative which contains a *Sst*I site instead of the *Hind*III site. For sequencing, the *Bam*HI–*Taq*I fragment was cloned in M13mp8 and 9 (ref. 23). Bm, *Bam*HI; HII, *Hind*II; HIII, *Hind*III; RI, *Eco*RI.

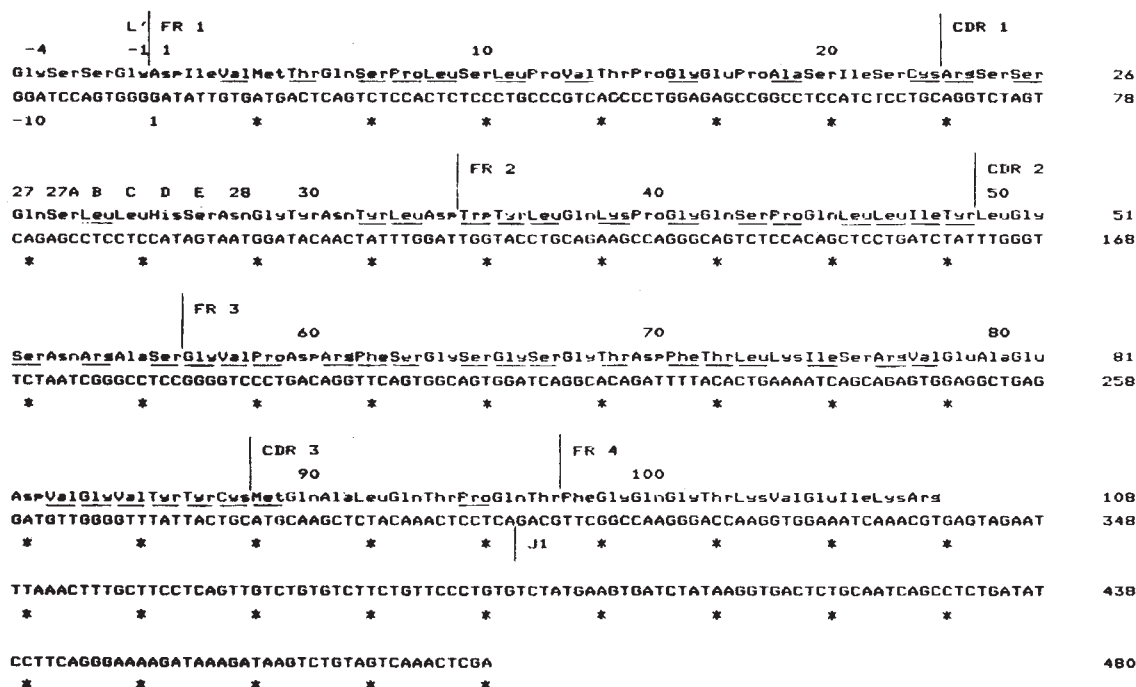


Fig. 2 Sequence of the V_{κ} – J_{κ} gene region of the rearranged allele of GM 607. The M13mp8 and mp9 subclones (Fig. 1) were sequenced by the chain terminator method²⁴ using 1-m long gels (ref. 25 and H. R. Jaenichen *et al.*, manuscript in preparation). Numbering of codons/amino acids is according to Kabat *et al.*⁶. V_{κ} specific amino acids are underlined. The J1 and intron sequence is identical to the sequence of Hieter *et al.*²¹ except for a GTG instead of a GTTG and a C instead of a T (positions 401–403 and 436, respectively, in our numbering). Regions: L', leader; FR, framework; CDR, complementarity determining.

Bence-Jones protein TEW¹⁵; there is 93% homology, if gaps are counted as one mismatch each and amide differences are included. Since our clone does not extend as far in the 5' direction as the region in which intron and leader sequences would be located, the assignment of codons –1 to –4 to amino acids of the leader remains tentative.

The sequence of the V_{κ} gene of GM 607 shares 64% homology with the published sequence of the $V_{\kappa 1}$ gene HK 102 (ref. 16) which has been confirmed and extended in our laboratory (H. R. Jaenichen *et al.*, manuscript in preparation). In this sequence comparison the 15 base pairs (bp) from position 82 to 96 which are not present in the $V_{\kappa 1}$ gene are counted as one

mismatch. The homologies between the complementarity determining regions of the $V_{\kappa 1}$ and $V_{\kappa 11}$ genes were found to be low: CDR1, 55%; CDR2, 33%; CDR3, 29%. Although the framework regions of the two genes show fairly high homologies (FR1, 62%; FR2, 80%; FR3, 76%) the degree of cross-hybridization was very low. In dot blot¹⁷ experiments (Fig. 3) 3% of cross-hybridization were found under stringent and 4–5% under non-stringent conditions. In Southern blot hybridizations¹⁸ cross-hybridization between the subgroup I and II genes was barely detectable.

To estimate the approximate number of germ-line $V_{\kappa 11}$ genes restriction nuclease digests of total human DNA were hybridized

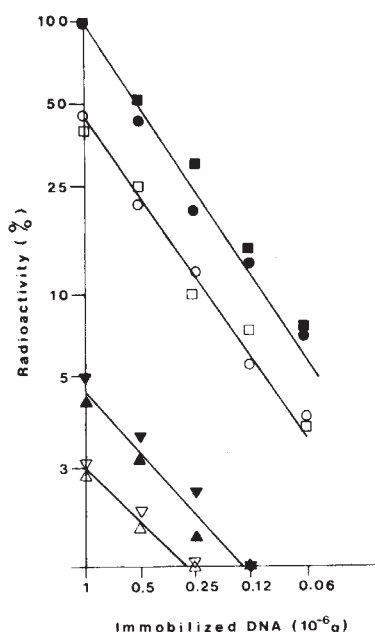


Fig. 3 Cross-hybridization between V_{κ} genes of subgroups I and II. The DNAs used were derived from the GM 607 subclone in M13mp9 (Fig. 1) ($V_{\kappa II}$) and from a $V_{\kappa I}$ clone in M13 phage. The inserts comprise 492 and 550 bp, respectively, the latter one being identical to the *Pst*I fragment of HK 102 (ref. 16; H. R. Jaenichen *et al.*, manuscript in preparation). The inserts were self-ligated to sizes of at least 5 kb. Approximately 0.06 to 1 μ g DNA each of the DNAs were applied to nitrocellulose paper and immobilized¹⁷. The same DNAs were nick-translated to about equal specific radioactivities with [α -³²P]dATP and [α -³²P]dCTP²⁶. Self- and cross-hybridizations were carried out in 6 $\times$ SSC at 68 °C; washing was with 6 $\times$ SSC or down to 0.1 $\times$ SSC (non-stringent and stringent, respectively). Individual dots were cut out (1 cm² each), counted, and the values plotted (after subtraction of blanks); non-stringent self-hybridizations (~4,000 c.p.m.) were taken as 100%. Self-hybridizations with $V_{\kappa I}$ DNA ($\circ$, $\bullet$) and $V_{\kappa II}$ DNA ($\square$, $\blacksquare$), cross-hybridizations with $V_{\kappa I}$ (Δ , $\blacktriangle$) and $V_{\kappa II}$ (∇ , $\blacktriangledown$) DNAs as probes; open and closed symbols represent stringent and non-stringent conditions.

under various conditions with the $V_{\kappa II}$ and, for comparison, the $V_{\kappa I}$ probes (Fig. 4). With the $V_{\kappa I}$ probe we obtained band patterns very similar to those of Bentley and Rabbitts⁵ who interpreted them to represent 15–20 V_{κ} genes. Such an interpretation is possible since the authors found only little polymorphism of restriction sites in the V_{κ} locus; they also took into account, as far as possible, the different intensities of the bands in the blot hybridization experiments. On the same basis we estimate that the $V_{\kappa II}$ subgroup contains about half as many genes as are detected with the $V_{\kappa I}$ probe. The strong bands of the $V_{\kappa II}$ patterns cannot represent $V_{\kappa I}$ genes because cross-hybridization between the two DNA probes is negligible.

Any figure for the number of different genes in the human V_{κ} locus which is based on the counting of bands in an electrophoretic separation can be taken only as an estimate. The sequence divergence within the subgroups could be rather high so that a subgroup-specific probe would detect extreme variants only as weak bands. The degree of cross-hybridization between the subgroups, on the other hand, is difficult to predict on the basis of amino acid or even nucleic acid sequences; this may be a source of both possible over- and underestimates of gene numbers. The presence of very homologous genes probably arising from recent gene duplication(s) in the V_{κ} locus, means that the actual number of V_{κ} genes detected by a subgroup I probe may be somewhat higher¹⁹ than originally proposed⁵. When several hybridization probes are available for each of the four subgroups it will be possible to make more exact estimates. The number of V_{κ} genes will be known definitely only after the

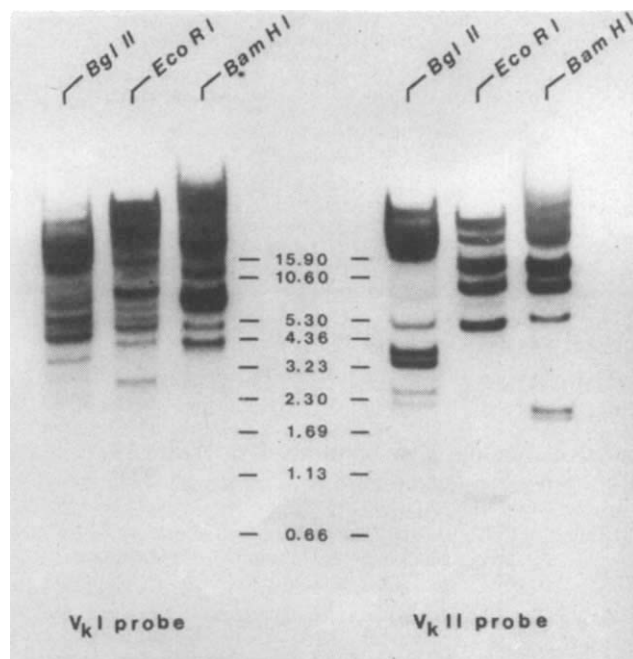


Fig. 4 Hybridization of restriction nuclease digests of total human DNA with $V_{\kappa I}$ and $V_{\kappa II}$ probes. 15 μ g each of *Bam*HI, *Eco*RI and *Bgl*II digests of human placenta DNA were separated electrophoretically on 6 mm thick 1% agarose gels in a buffer (similar to ref. 27), 40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4. After blotting¹⁸ hybridization with the nick-translated probes (Fig. 3) was in 3 $\times$ SSC at 68 °C and washing in 2 $\times$ SSC. Autoradiography was without intensifying screens.

whole V_{κ} locus has been cloned. Since only sequencing can distinguish between potentially functional genes and pseudogenes, the exact determination of the functional V_{κ} gene repertoire is a long-term project. Present estimates, however, clearly indicate that in humans the V_{κ} gene repertoire is relatively small and that other mechanisms, for example, somatic mutation, must contribute significantly to the generation of the V_{κ} -sequence diversity.

After submission of the present report, a paper was published²⁸ describing the sequence and hybridization properties of a $V_{\kappa III}$ cDNA. The results are interpreted to be in agreement with Bentley and Rabbitts⁵ and 50 or less germ-line V_{κ} genes are proposed.

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1. Tonegawa, S. *Nature* **302**, 575–581 (1983).
2. Honjo, T. A. *Rev. Immun.* **1**, 499–528 (1983).
3. Valbuena, O., Marcu, K. B., Weigert, M. & Perry, R. P. *Nature* **276**, 780–784 (1978).
4. Cory, S., Tyler, B. M. & Adams, J. M. *J. molec. appl. Genet.* **1**, 103–116 (1981).
5. Bentley, D. L. & Rabbitts, T. H. *Cell* **24**, 613–623 (1981).
6. Kabat, E. A., Wu, T. T., Bilofsky, H., Reid-Miller, M. & Perry, H. *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, 1983).
7. Pech, M., Höchtl, J., Schnell, H. & Zachau, H. G. *Nature* **291**, 668–670 (1981).
8. Gearhart, P. J. & Bogenhagen, D. F. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3439–3443 (1983).
9. Baltimore, D. *Cell* **26**, 295–296 (1981).
10. Milstein, C. *Nature* **216**, 330–332 (1967).
11. Hood, L. & Talmage, D. W. *Science* **168**, 325–334 (1970).
12. Wang, A.-C., Fudenberg, H. H., Wells, J. V. & Roelcke, D. *Nature new Biol.* **243**, 126–127 (1973).
13. Solomon, A. *J. clin. Invest.* **61**, 97–108 (1978).
14. Dolby, T. W., Devuono, J. & Croce, C. M. *Proc. natn. Acad. Sci. U.S.A.* **77**, 6027–6031 (1980).
15. Putnam, F. W., Whitley, E. J. Jr, Paul, C. & Davidson, J. N. *Biochemistry* **12**, 3763–3780 (1973).
16. Bentley, D. L. & Rabbitts, T. H. *Nature* **288**, 730–733 (1980).

17. Kafatos, F. C., Jones, C. W. & Efstratiadis, A. *Nucleic Acids Res.* **7**, 1541-1552 (1979).
18. Southern, E. M. *J. molec. Biol.* **98**, 503-517 (1975).
19. Bentley, D. L. & Rabbitts, T. H. *Cell* **32**, 181-189 (1983).
20. Rimm, D. L., Horness, D., Kucera, J. & Blattner, F. R. *Gene* **12**, 301-309 (1980).
21. Hieter, P. A., Maizel, J. V. Jr & Leder, P. *J. biol. Chem.* **257**, 1516-1522 (1982).
22. Neumaier, P. S. & Zachau, H. G. *Nucleic Acids Res.* **11**, 3631-3636 (1983).
23. Messing, J. & Vieira, J. *Gene* **19**, 269-276 (1982).
24. Sanger, F., Coulson, A., Barrell, B., Smith, A. & Roe, B. *J. molec. Biol.* **143**, 161-178 (1980).
25. Garoff, H. & Ansorge, W. *Analyt. Biochem.* **115**, 450-457 (1981).
26. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. *J. molec. Biol.* **113**, 237-251 (1977).
27. Loening, U. E. *Biochem. J.* **102**, 251-257 (1967).
28. Bentley, D. L. *Nature* **307**, 77-80 (1984).

Isolation of a cDNA clone coding for an SB β -chain

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Class II antigens of the major histocompatibility complex (MHC) consist of a family of closely related cell surface-expressed glycoproteins. These antigens, which are genetically polymorphic, control important aspects of the immune response¹. At least three types of human class II antigens, namely, DR, DC and SB (refs 2-4), have been identified. All class II antigens are heterodimers composed of one α - and one β -chain. The genes for both types of subunits are encompassed within the MHC^{5,6}. The general features of the DC and DR antigens have recently been elucidated⁷⁻¹⁵. Much less is known, however, about the SB molecules¹⁶. Here we describe the isolation of a cDNA clone as well as a genomic clone encoding a β -chain whose amino acid sequence is compatible with the partial amino-terminal sequence of SB β -chains¹⁶.

Recent studies have shown that part of the 3'-untranslated regions of DR and DC cDNA clones can be used as locus-specific probes in hybridization experiments¹⁷. This information

prompted us to isolate non-DR, non-DC locus-derived cDNA clones from a previously described library¹⁸. Colony hybridizations were carried out separately using probes corresponding to the translated and the 3'-untranslated portions of DR and DC β cDNA clones, respectively^{12,19}. One cDNA clone, pII- β -7, hybridized to the probes corresponding to the translated portions but did not hybridize to the 3'-untranslated probes. Therefore, pII- β -7 was characterized by restriction mapping and nucleotide sequence analysis. Figure 1 summarizes the results and displays the amino acid sequence predicted from the nucleotide sequence; the data demonstrate that pII- β -7 is truncated in the 5' region as only three of the four cysteine codons, characteristic for all β -chains sequenced so far^{9,10,12,14} are present in the clone (compare Fig. 2).

To deduce the amino-terminal sequence of a β -chain of the same type as that encoded by pII- β -7, we isolated a genomic clone from a DR/Dw 4/4 cosmid library (B. Serenius *et al.*, in preparation) with this cDNA clone as the probe. This cosmid clone, cosII-412, contains a β gene that is related to pII- β -7 according to the following criteria: the 3'-untranslated sequences of the two clones show 90% homology (data not shown). No homology whatsoever is found between the 3'-untranslated portions of these clones and clones encoding DR and DC β -chains. The exon encoding the first domain of cosII-412 is highly homologous to the portion of pII- β -7 available for comparison. Also, the amino acid sequences predicted from the two clones are homologous (Fig. 1b). The six nucleotide substitutions between the sequenced portions of cosII-412 and pII- β -7 (derived from a DR 3/w6 library) are not unexpected as the polymorphism of the β -chains is almost exclusively localized in the first domain¹⁰.

pII- β -7 and cosII-412 encode β -chains that are distinct from, but homologous to, DR and DC β -chains (Fig. 2). To obtain maximal homology with the latter sequences, a gap of two residues had to be introduced into the amino acid sequence predicted from cosII-412. Accordingly, the acceptor site of the first splice junction in cosII-412 resides in the same codon (+5) as in DC β (ref. 20) and DR β genes (D. Larhammar *et al.*, in preparation), assuming that the same number of amino acids are encoded by the signal sequence exons. The extracellular portions of the β -chains corresponding to pII- β -7 and cosII-412

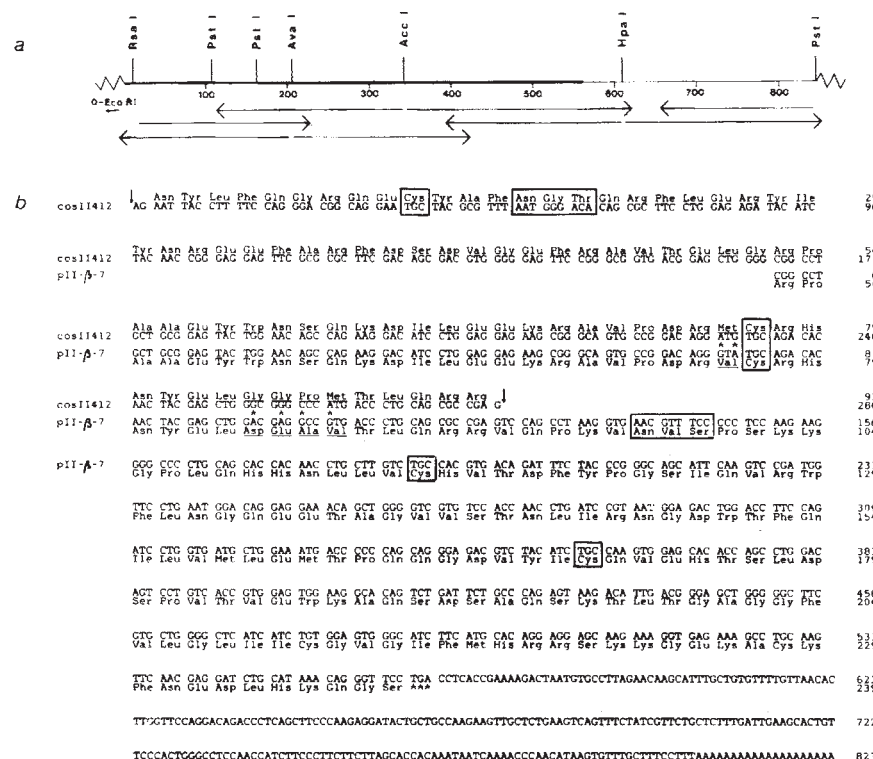


Fig. 1 a, Restriction map of the pII- β -7 cDNA insert, with the translated portion indicated by a thick line. The shortest distance from the cloning site (*Pst*I) to the *Eco*RI site at position 0 in pBR322 is indicated. The sequencing strategy is shown by arrows. b, The nucleotide and predicted amino acid sequences of the cDNA insert of pII- β -7 and of the first domain exon of cosII-412. Asterisks denote nucleotide substitutions between the two sequences. Amino acid replacements are underlined. The nucleotide sequence was determined according to the procedure of Maxam and Gilbert²². Cysteine residues, which by homology to other class II antigen β -chains are disulphide-linked, are boxed, as are two putative attachment sites for N-linked oligosaccharides at Asn 19 and Asn 98. The first cysteine residue is numbered 15 in accordance with DR and DC β sequences. cosII-412 was isolated from a genomic library consisting of DNA from an Dw/DR 4/4 individual partially digested with *Mbo*I and inserted into the cosmid vector pHEP (ref. 23). pII- β -7 was used as the probe in colony hybridization. cosII-412, which hybridized strongly to the probe, contained the first domain exon in a 7.3-kb *Hind*III fragment, which was subcloned into pUC9 and used for nucleotide sequencing.

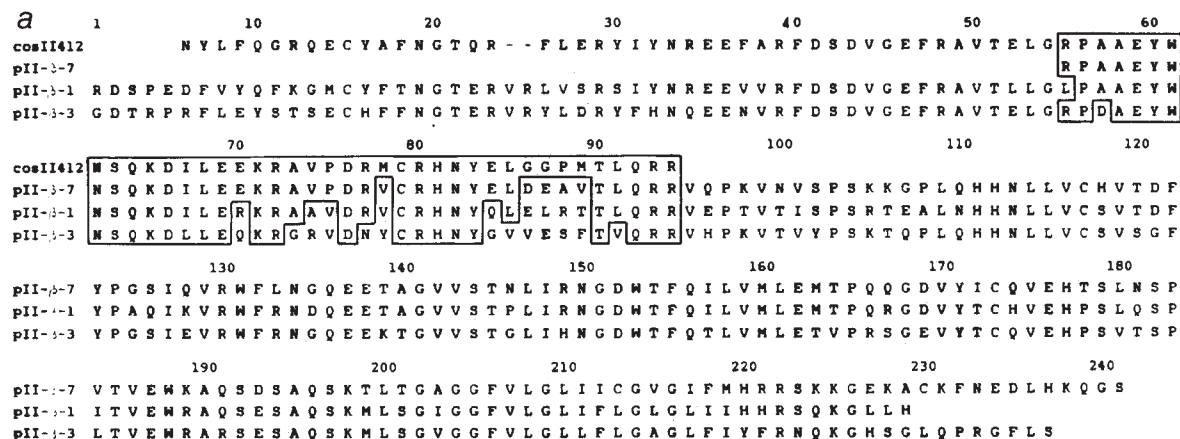


Fig. 2 a, Comparison of the amino acid sequences predicted from pII-β-7 and cosII-412 with the corresponding sequences of pII-β-1 (DC)¹¹ and pII-β-3 (DR)¹⁹. A gap of two residues was introduced into the sequence predicted from cosII-412 to maximize the homology. Amino acid residues homologous to the cosII-412 β-chain in the portion where this sequence overlaps the pII-β-7 β-chain sequence, are boxed. **b**, Comparison of the partial amino-terminal sequence of an SB β-chain¹⁶ with the predicted amino acid sequences of cosII-412, cosII-102 (ref. 20), pII-β-1 (ref. 12), pII-β-2 (ref. 10), pII-β-3 and -4 (ref. 19) and HLA-DRβ1 (ref. 14). Tyrosine and phenylalanine residues are enclosed in open or shaded boxes. Shaded residues coincide with the tyrosines and phenylalanines present in the SB β sequence.

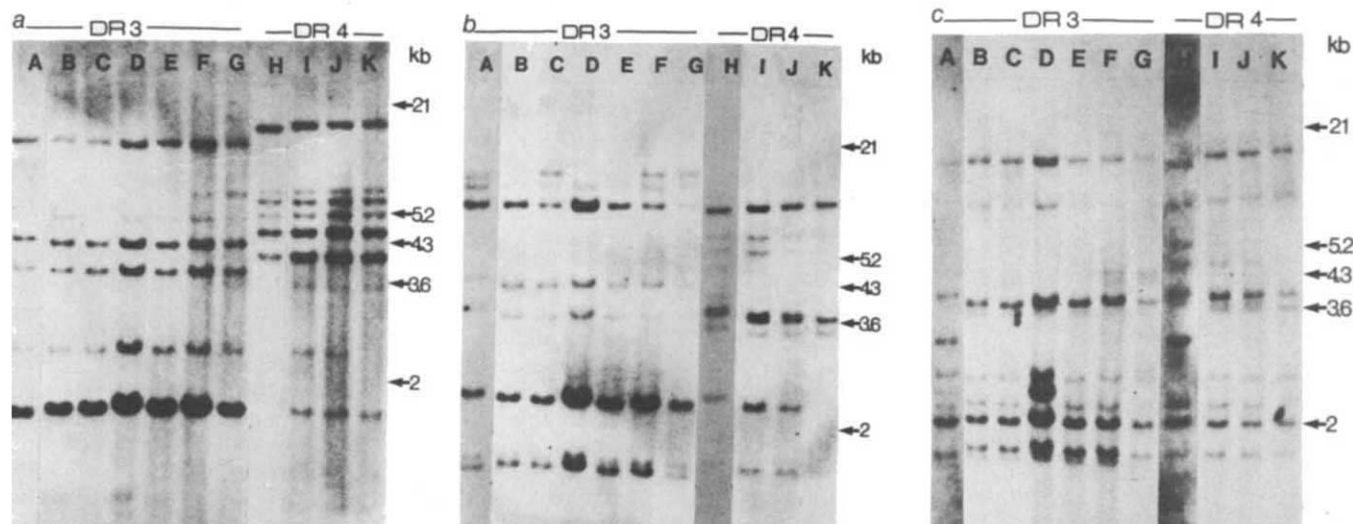
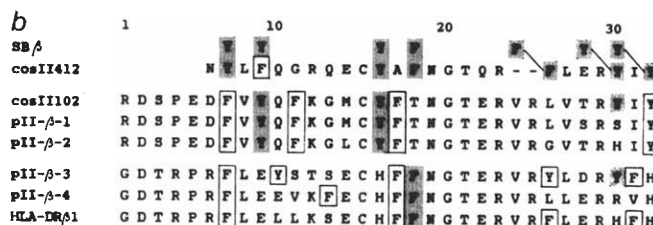


Fig. 3 Hybridizations of probes corresponding to the translated part of DR (**a**) and DC (**b**) β-chain cDNA clones as well as pII-β-7 (**c**) to *Pvu*II-digested genomic DNA from homozygous donors of specificities Dw/DR 3/3 and 4/4. The DC β probe was isolated from pII-β-1 after digestion with *Ava*I, the DR β probe was isolated from pII-β-3 (ref. 19) after digestion with *Sac*I and *Hind*III, and the pII-β-7 probe was isolated after cleavage with *Rsa*I and *Hpa*I (Fig. 1). All probes are derived from the translated regions of the three β cDNA clones. The probes were nick-translated (ref. 24) to a specific activity of $5\text{--}10 \times 10^7$ c.p.m. μg^{-1} . DNA was isolated from the cells as described previously²⁵. DNA samples (10 μg) were digested to completion with *Pvu*II (New England Biolabs) and run on 0.7% agarose gels in 89 mM Tris-borate buffer, pH 8.3 containing 2.6 mM EDTA for 600 volt-hours and transferred to nitrocellulose filters²⁶. The filters were prehybridized for 3–6 h at 42 °C in 50% formamide, 5 × SSPE buffer (SSPE = 10 mM sodium phosphate pH 7.0, containing 1 mM EDTA, 180 mM NaCl), 5 × Denhardt's solution²⁷, 0.02% of salmon sperm DNA and 0.2% SDS. Radioactively labelled cDNA fragments were added ($1\text{--}2 \times 10^6$ c.p.m. ml^{-1}) in a solution of 50% formamide, 5 × SSPE buffer, 1 × Denhardt's solution, 0.02% salmon sperm DNA, 0.2% SDS and 10% dextran sulphate. After hybridization at 42 °C for 16–24 h, the filters were washed twice in 2 × SSPE buffer, 0.2% SDS for 5 min at room temperature and twice in 0.2 × SSPE buffer, 0.2% SDS for 20 min at 55 °C. The filters were then autoradiographed with intensifying screens at –70 °C.

display approximately 67 and 71% homology to DR and DC β -chain, respectively. The latter two types of β -chains are approximately 66% homologous with each other (Fig. 2a). Thus, pII- β -7 and cosII-412 encode a new type of class II antigen β -chain. This is also evident from the predicted structure of the cytoplasmic portion of the pII- β -7 β -chain; it consists of 22 amino acids in contrast to the cytoplasmic portions of DR¹⁴ and DC^{10,12,20} β -chains, which are composed of 16 and 10 residues, respectively.

A partial amino-terminal sequence of an SB β -chain has been determined, locating the positions of five tyrosine and two phenylalanine residues¹⁶. Except for one tyrosine, all these residues coincided with tyrosines and phenylalanines in the amino acid sequence predicted from cosII-412 (Fig. 2b). Moreover, no additional residues of these types were found in the sequence. In contrast, of the seven residues identified in the SB β -chain, only two (or three) tyrosines coincided with tyrosines in DC β -chains and only one phenylalanine with the corresponding residue in DR β -chains. One DR β -chain sequence also displayed one of the tyrosines found in the SB β -chain. The DC and DR β -chains also contained additional tyrosine and phenylalanine residues at the amino terminus. We suggest, therefore, that cosII-412 and pII- β -7 encode SB β -chains (or chains closely related to SB β).

The SB locus is located at some distance towards the centromere from the DR and DC loci, which are tightly linked⁴. To study the degree of linkage between the gene corresponding to pII- β -7 and the DR locus, we used pII- β -7 as a hybridization probe to genomic DNA of HLA D/DR homozygous individuals. For comparison, hybridizations were also carried out with DR and DC β -chain probes. The DNA samples (seven of which were typed to be Dw/DR 3/3 and four to be Dw/DR 4/4) were digested with *Pvu*II. The experimental conditions chosen for the hybridizations were such that no, or only slight, cross-hybridizations occurred with the three probes. Figure 3 shows that the banding patterns obtained were distinct and different. The probe corresponding to the translated portion of a DR β -chain clone gave rise to virtually identical patterns for all the DR 3/3 homozygous samples (Fig. 3a). It should be noted that the faint band of ~6 kilobases (kb) was present in all samples and that the figures are composites from several autoradiograms, thus making perfect alignments of the samples difficult to achieve. The DR 4/4 samples gave a pattern distinct from that of the DR 3/3 samples. However, apart from a weakly hybridizing band of 2.2 kb which was absent from one sample (Fig. 3a, lane K) all DR 4/4 homozygous samples appeared identical.

The DC β probe also gave rise to hybridization patterns that conformed well to the typed DR specificity of the sample. Thus, all seven DR 3/3 homozygous samples appeared identical, apart from one sample lacking a 9.2-kb band (Fig. 3b, lane D). This result agrees with the observation that the hybridization patterns of DR 4/4 homozygous samples, although different from those of the DR 3/3 samples, were also identical to each other. It is obvious that the 2.2-kb band, absent from lane K of Fig. 3b, was the result of cross-hybridization of the DC and DR β -chain probes to the same genomic sequence (compare Fig. 3a, lane K).

The hybridization patterns obtained with pII- β -7 as the probe were quite similar for the DR 3/3 and DR 4/4 samples (Fig. 3c). In addition, the variability between identical DR samples was of the same magnitude as between the DR 3/3 and DR 4/4 samples. Thus, the hybridization patterns did not correlate well with the DR specificity of the samples. This indicates that the locus corresponding to pII- β -7 and cosII-412 is located at some distance from the DR locus. This finding is perfectly compatible with the structural similarity between the β -chain encoded by cosII-412 and an isolated SB β -chain discussed above. We conclude, therefore, that pII- β -7 and cosII-412 code for SB β -chains (or chains closely related to SB β -chains).

After submission of this manuscript, Roux-Dosseto *et al.* reported the identification and sequence of a cDNA clone suggested to correspond to an SB β -chain²¹. The β -chain predicted from this clone is highly homologous to that deduced from

pII- β -7, except in the cytoplasmic portion, which only consists of 12 residues. Whether the clone described by Roux-Dosseto *et al.*²¹ is derived from the same gene as the clones described here remains to be investigated.

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1. Möller, E. *Immun. Rev.* **66**, 5-187 (1982).
2. Tosi, R., Tanigaki, T., Centis, D., Ferrara, G. B. & Pressman, D. J. *Expl. Med.* **148**, 1592-1611 (1978).
3. Shaw, S., Kavathas, P., Pollack, M. S., Charnot, D. & Mawas, C. *Nature* **293**, 745-749 (1981).
4. Terasaki, E. P. (ed.) *Histocompatibility Testing 1980*. (UCLA Tissue Typing Lab., Los Angeles, 1980).
5. Trowsdale, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 1972-1976 (1983).
6. Hood, L., Steinmetz, M. & Malissen, B. A. *Rev. Immun.* **1**, 529-568 (1983).
7. Auffray, C. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 6337-6341 (1982).
8. Korman, A. J., Auffray, C., Schamböeck, A. & Strominger, J. L. *Proc. natn. Acad. Sci. U.S.A.* **79**, 6013-6017 (1982).
9. Kratzin, H. *et al. Hoppe Seyler's Z. physiol. Chem.* **362**, 1665-1669 (1981).
10. Schenning, L. *et al. EMBO J.* **3**, 447-452 (1983).
11. Larhammar, D. *et al. Cell* **30**, 153-161 (1982).
12. Larhammar, D. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 3687-3691 (1982).
13. Lee, J. S. *et al. Nature* **299**, 750-752 (1982).
14. Long, E. O., Wake, C. T., Gorski, J. & Mach, B. *EMBO J.* **2**, 389-394 (1983).
15. Yang, C.-Y. *et al. Hoppe Seyler's Z. physiol. Chem.* **363**, 671-676 (1982).
16. Hurley, C. K., Shaw, S., Nadler, L., Schlossman, S. & Capra, J. D. *J. exp. Med.* **156**, 1557-1562 (1982).
17. Long, E. O. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 7465-7469 (1982).
18. Wiman, K. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 1703-1707 (1982).
19. Gustafsson, K. *et al. EMBO J.* (submitted).
20. Larhammar, D. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 7313-7317 (1983).
21. Roux-Dosseto, M. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 6036-6040 (1983).
22. Maxam, A. M. & Gilbert, W. *Meth. Enzym.* **65**, 499-560 (1980).
23. Grosfeld, F. G. *et al. Nucleic Acids Res.* **10**, 6715-6732 (1982).
24. Rigby, P. W. J., Diekmann, M., Rhodes, C. & Berg, P. *J. molec. Biol.* **113**, 237-251 (1977).
25. Böhme, J. *et al. Nature* **301**, 82-84 (1983).
26. Southern, E. J. *J. molec. Biol.* **98**, 503-517 (1975).
27. Denhardt, D. T. *Biochem. biophys. Res. Commun.* **23**, 641-646 (1966).

Immortalization of monkey epithelial cells by specific fragments of Epstein-Barr virus DNA

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Epstein-Barr virus (EBV) is unique among the DNA tumour viruses by virtue of its association with two human malignancies, Burkitt's lymphoma and nasopharyngeal carcinoma (NPC), the former a tumour of B lymphocytes and the latter encompassing low-differentiated epithelial cells of the nasopharynx¹⁻⁴. A viral gene product has not been definitively linked to these malignant diseases, although an EBV nuclear antigen(s) (EBNA) seems to be ubiquitous in EBV-infected cells; indeed, the detection of EBNA by immunofluorescence is often taken as an indication of the presence of the viral genome^{5,6}. As part of a study to investigate which part of the EBV genome is responsible for transformation and whether the same mechanism of cellular transformation is involved in the case of B lymphocytes and epithelial cells, we have tried to establish whether a detectable cellular alteration(s) can be induced in primate epithelial cells by the presence of a specific region of the EBV genome. We report here that it can—the result is immortalization of the cells.

A hybrid cosmid library was made that encompasses the viral genome within six fragments (see Fig. 1), using partially cleaved *Bam*HI fragments from B95-8 virion DNA cloned into the cosmid pHC79 (ref. 7). These recombinants, together with virion and episomal⁸ EBV DNAs, and suitable controls, were used to transfect a mixture of cells derived from African green monkey (*Cercopithecus aethiops*) kidneys (AGMK cells). After

~3 months in culture, very small, densely staining cell populations were observed in several of the experiments but the two dishes that contained cells transfected with DNA from cosmid clones designated p13/p33 and p31 were readily distinguishable from the others by the quantity and size of 'foci' in them. Details of the transfection experiments, and data derived from them, are given in Fig. 2. The results illustrated in Fig. 2 were confirmed by examination of unstained dishes under the light microscope.

Individual foci were selected from AGMK cells transfected with p13/p33 and p31 DNAs, as well as from dishes containing cells transfected with either calf thymus (control) or clone p65 DNAs. Foci were transferred manually to fresh dishes (50 mm) and fed weekly. Whereas none of the small foci from the calf thymus or p65 experiments grew, nine cell lines were established from foci (out of the 10 selected from each experiment) derived from the p13/p33 or the p31 dishes. Thus, only AGMK cells exposed to EBV DNA fragments from a specific region of the viral genome could be propagated in culture. Several of the cell lines have been studied further. Five of them subdivided at a 1:10 dilution weekly have been kept for more than a year in culture (corresponding to at least 200 generations) with no apparent change in morphology or growth rate, as determined by regular examination by light microscopy. By morphological and histological examination the cells have been shown to be epithelial in type (see Fig. 3a-c). Cells from the 13/33 f4 and the 31 f4 experiments, screened by immunofluorescence, show the presence of intermediate filaments typical of epithelial cells^{9,10} (see Fig. 3d, e). Whereas microscopic examination of the original AGMK cells revealed cells characteristic of elongated and cuboidal epithelium, and of fibroblasts, the immortalized cells exhibited only the simple cuboidal morphology (Fig. 3b, c). Thus, it seems that a subpopulation of monkey epithelial cells has been immortalized by transfection with the EBV DNA fragments.

All cell lines examined proved to be capable of growing for a limited time in low serum. Cell growth in either normal (5%) or low (0.5%) concentrations of fetal calf serum (FCS) was independent of serum concentration during the first month in culture, cells being subdivided at a 1:10 dilution weekly and cell counts determined. After 2 months, the growth rate in low serum slowed to about one-half that of the cells growing in normal serum. Qualitatively similar results were obtained when the cells were plated into semi-solid (agar) media, that is, initially the cells divided and small visible colonies, containing a number of greatly enlarged cells, were observed within 3-4 weeks. These colonies generally then ceased to undergo further division, but on being isolated and re-plated onto plastic, they resumed division and grew normally. In additional experiments, cells from early passages of a number of the lines, as well as control AGMK cells, were injected intraperitoneally into young nude mice (10^6 cells per animal); no tumours were apparent a year later. (Similar experiments using late passage cells are in progress.) These observations all indicate some dramatic alteration in the growth properties of the cells, effected by specific fragments of EBV DNA, that are not sufficient, however, to produce a malignant phenotype.

As a permanent change seemed to have been produced in the AGMK cells following transfection with either of two defined, partly overlapping fragments of the EBV genome, it remained to be determined whether the continued presence of the virus-derived DNA could be observed. Therefore, total chromosomal DNA from four of the established cell lines was applied directly to nitrocellulose filters and probed with either ³²P-labelled B95-8 virion DNA, or cloned fragments of EBV DNA, in order to detect viral DNA sequences. The presence of EBV-related sequences was observed in all four cell lines analysed, but not in the calf thymus DNA used as a control (data not shown). To examine further the nature of EBV DNA sequences in the cell lines, cellular DNA was cleaved with the *Bam*HI restriction enzyme and the resulting fragments were analysed by established methods¹¹. Specific fragments that hybridized to viral DNA were observed (Fig. 4); the patterns

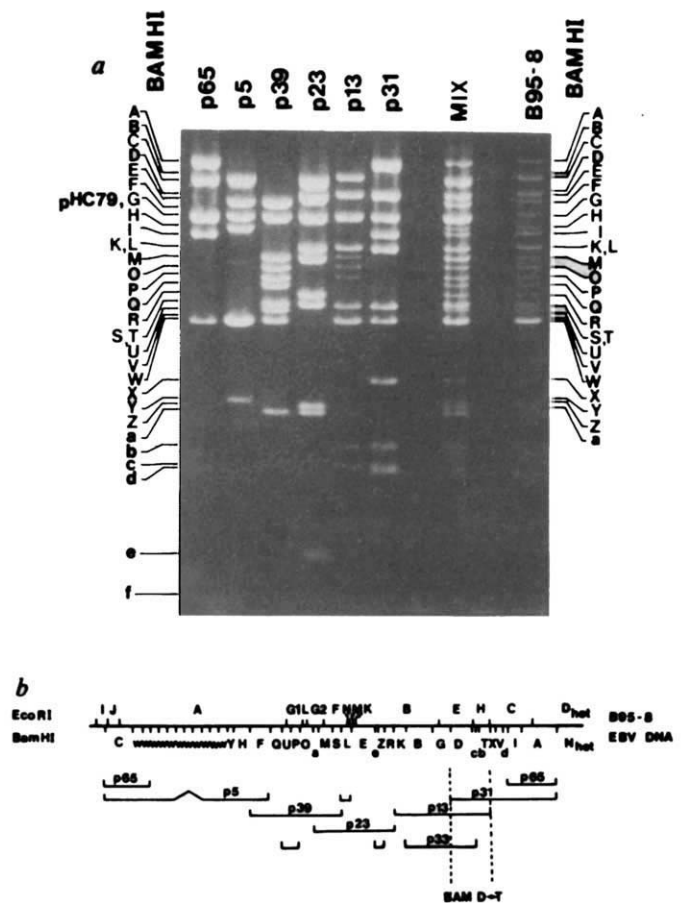


Fig. 1 *a*, Cloned large fragments of EBV DNA. Virion EB DNA²² (5 µg) from B95-8 marmoset cells was partially digested with *Bam*HI, and fractionated by neutral sucrose density gradient centrifugation to reduce contamination with small fragments. DNA fragments of ~40 kilobases (kb) were ligated to the *Bam*HI-cleaved cosmid pHC79 DNA (which had been dephosphorylated with alkaline phosphatase), and the resulting hybrid DNAs were cloned according to the procedure of Hohn²³. Isolated clones were initially analysed by the 'dot-blot' method using individually the large *Eco*RI fragments A to G (ref. 24), radioactively labelled with ³²P according to Rigby *et al.*²⁵, as probes. DNAs from clones that yielded results consistent with the known physical map of EBV DNA were further analysed by electrophoresis on 0.8% agarose gels in Tris-acetate buffer in the presence of ethidium bromide and visualized with UV light, after treatment with *Bam*HI. A 'library' was selected, from among 780 clones analysed, which consisted of six clones designated p65, p5, p39, p23, p13 and p31, as shown. A mixture of the cloned DNAs (MIX) produced a pattern that was qualitatively indistinguishable from the viral (B95-8) DNA. The location of known fragments (A to e) and pHC79, which co-migrates with *Bam*HI G, are indicated; the presence of the latter was confirmed by hybridization against DNA from a clone of *Bam*HI G fragment (data not shown). In addition, a previously unknown fragment, *Bam*HI f, was observed in the clone p23. (The p13 clone, shown here, contained an impurity which was subsequently removed by standard procedures before proceeding to the transfection studies.) *b*, Schematic diagram showing the location of DNA from cosmid clones p65, p5, p39, p23 and p31 relative to the *Eco*RI and *Bam*HI physical maps of the 170-kb linear EBV virion DNA^{26,27}. The location of DNA from another clone, p33, is also indicated. (An original 'mini-prep' analysis of clone p33 mistakenly suggested that it contained *Bam*HI R; it was therefore used in transfection experiments (Fig. 2) together with p13 in order to provide overlap information with p23. Its corrected sequence, based on hybridization analysis, is shown.) The dashed vertical lines indicate the 9-kb sequence (*Bam* D→T) which clones p13 and p31 have in common. Contiguous sequences from these two clones comprise about one-third of the viral genome.

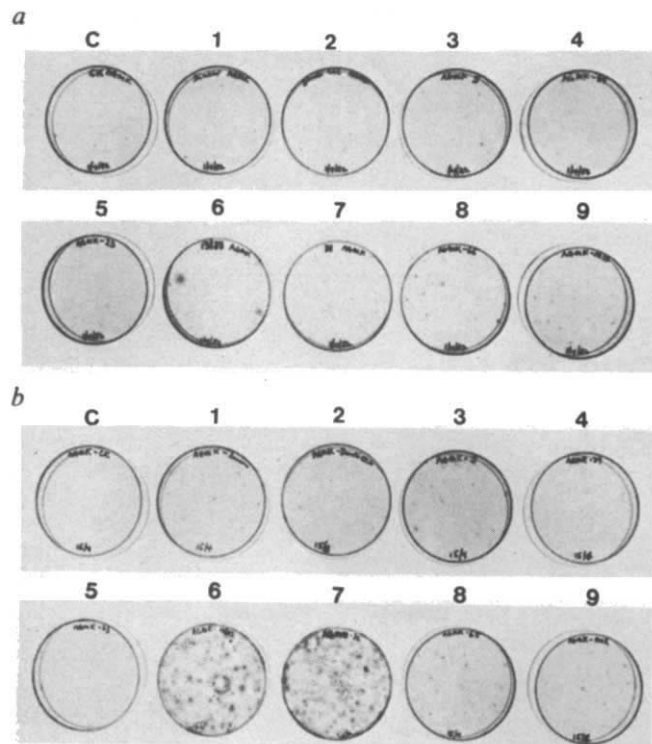


Fig. 2 Dense focus assay of AGMK cells exposed to different fragments of EBV DNA. Subconfluent dishes (50 mm) of early passage AGMK cells were transfected with EBV DNA as indicated below, in the presence of calf thymus carrier DNA ($5 \mu\text{g}$ per dish) and calcium phosphate²⁸. Cells were left for 6 h at 37°C in Dulbecco's modified Eagle's medium (DMEM)-3% glutamine-5% fetal calf serum, then the medium was removed and fresh medium added. After 6 weeks in culture, with weekly changes of medium, cells on each dish were trypsinized and re-plated at a 1:5 dilution onto 50-mm dishes. Thereafter, cells were grown at 37°C and the media were changed weekly. Individual dishes shown contained AGMK cells transfected with calf thymus (carrier) DNA alone (C) or with linear virion DNA²² (1); episomal DNA⁸ from Daudi cells (2); DNA from clones p5 (3), p39 (4), p23 (5), p13+p33 (6), p31 (7), and p65 (8); and a mixture of cloned DNAs (9). After subculturing, the dishes were left for 4 weeks in culture and 'foci' (a) were visualized using Leishman's stain. Whereas aggregations of cells or minor foci were observed at this time in a number of cases, notably among those cells transfected with DNA from the clone p65, p31 or with a mixture of all the cloned DNAs, large distinct foci were observed only in the dish of AGMK cells transfected with the p13/p33 DNA clones. After a further 2 weeks in culture (b), large densely growing cell colonies were evident in both the p13/p33 (6) and p31 (7) experiments. EBV DNA was used at a concentration of $5 \mu\text{g}$ per dish, except in the 'mix' experiment (9), where the total amount of viral DNA was $\sim 25 \mu\text{g}$. Failure to form foci in the latter experiment may be a consequence of 'poison sequences' in the cosmid at the concentration used²⁹.

obtained were not observed when uncleaved material was subjected to similar analyses. In very early passage cultures of 13/33 f4 cells, four bands hybridized to viral DNA (Fig. 4a); in later passages of the same cell line, the two largest bands were replaced by a single band having an electrophoretic mobility intermediate between the *Bam*HI co-migrating fragments B/C and D/E (see Fig. 4b-d). No further change in the DNA was observed in cells carried for more than a year *in vitro*. These data suggest that some sequence alteration may have occurred between the initial integration event(s) and the establishment of the cells in culture. The two smallest fragments initially observed (Fig. 4a) may be derived from monkey DNA as, unlike the larger fragments, their hybridization with EBV DNA could

be competitively inhibited by prehybridization with excess cold AGMK DNA. Although cross-hybridization with monkey DNA has not previously been reported, it is well documented that Epstein-Barr virion DNA has regions of homology with a variety of other eukaryotic DNAs^{12,13}.

Examination of the 13/33 and 31 cell lines for the expression of EBNA proved negative, even though the genetic information for this antigen could at least theoretically have been present in the 13/33 lines (but not in the 31 lines)¹⁴. Negative results for EBNA were also obtained in the laboratories of G. Klein and D. Crawford (personal communications).

The results of our experiments are consistent with the notion that a specific sub-genomic region of the Epstein-Barr viral

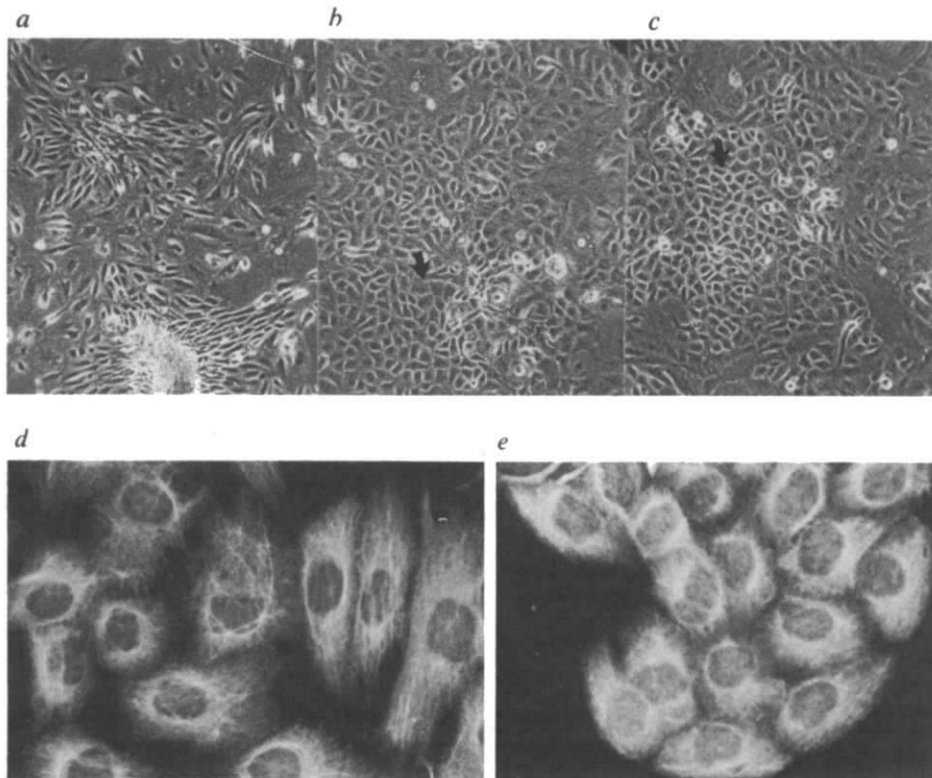


Fig. 3 Morphology of immortalized cells. a-c, Light micrographs of a heterogeneous population of cells observed in an early culture of AGMK cells (a) and in dishes containing the cuboidal epithelial cells present in the line 13/33 f4 after 14 (b) or 34 (c) weeks in culture. 13/33 f4 cells examined at an earlier time (4 weeks) showed no obvious morphological differences from the 14- or 34-week cultures. Living cells shown by phase-contrast optics. $\times 48$. d, e, Immunofluorescent staining with the monoclonal antibody LE61 which recognizes kidney epithelium⁹, showing the tonofilament organization of stained cells. Subconfluent cells from the 31 f4 cell line (d) are shown, together with the typical close-packing arrangement of rapidly growing cells (e), as exemplified by cells from the 13/33 f4 line.

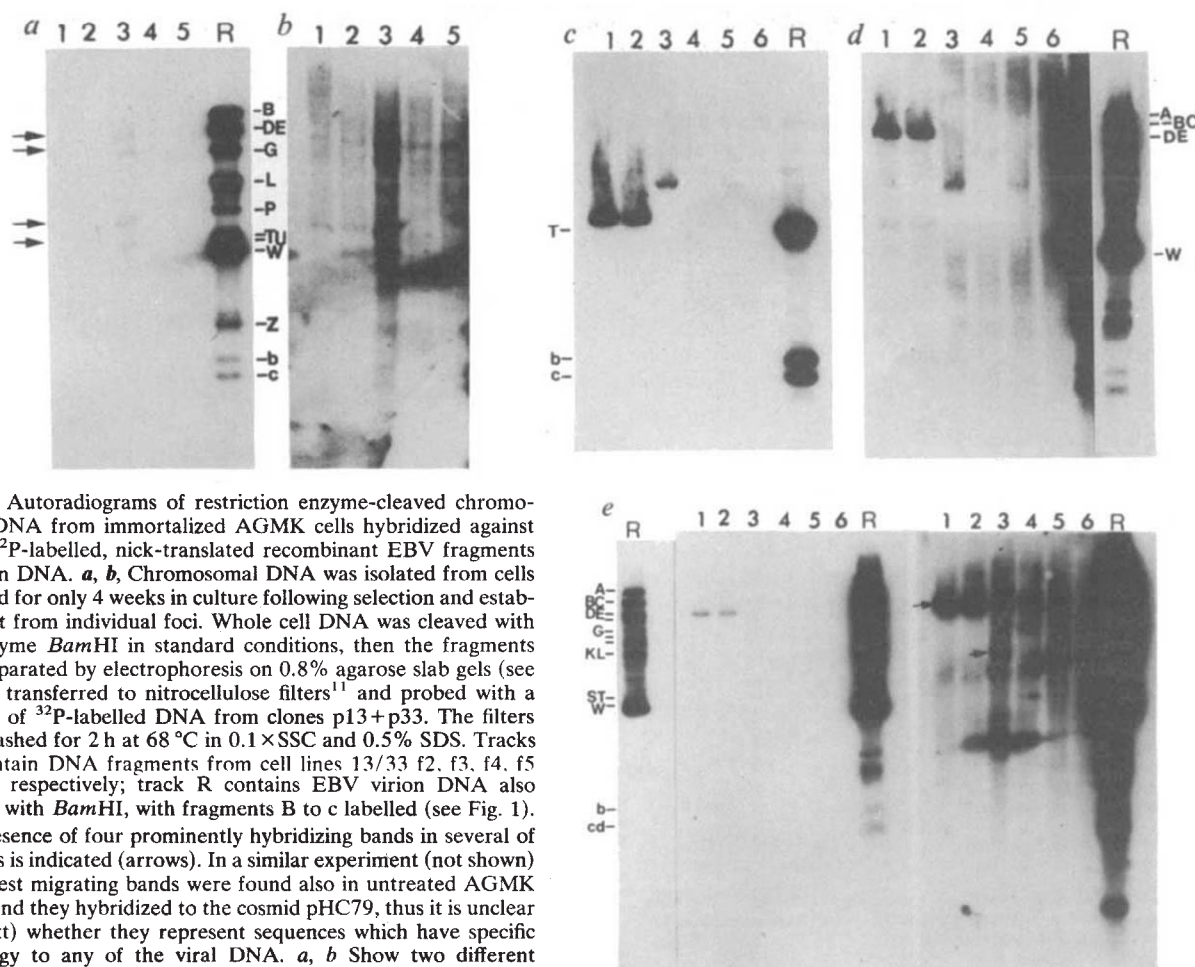


Fig. 4 Autoradiograms of restriction enzyme-cleaved chromosomal DNA from immortalized AGMK cells hybridized against either 32 P-labelled, nick-translated recombinant EBV fragments or virion DNA. **a, b**, Chromosomal DNA was isolated from cells passed for only 4 weeks in culture following selection and establishment from individual foci. Whole cell DNA was cleaved with the enzyme *Bam*HI in standard conditions, then the fragments were separated by electrophoresis on 0.8% agarose slab gels (see Fig. 1), transferred to nitrocellulose filters¹¹ and probed with a mixture of 32 P-labelled DNA from clones p13+p33. The filters were washed for 2 h at 68 °C in 0.1×SSC and 0.5% SDS. Tracks 1–5 contain DNA fragments from cell lines 13/33 f2, f3, f4, f5 and f6, respectively; track R contains EBV virion DNA also cleaved with *Bam*HI, with fragments B to c labelled (see Fig. 1).

The presence of four prominently hybridizing bands in several of the lines is indicated (arrows). In a similar experiment (not shown) the fastest migrating bands were found also in untreated AGMK DNA, and they hybridized to the cosmid pH79, thus it is unclear (see text) whether they represent sequences which have specific homology to any of the viral DNA. **a, b** Show two different time exposures. In the longer exposure (**b**), minor bands can be observed, particularly in track 3 which corresponds to material from the cell line 13/33 f4. **c, d**, Analysis of DNA from cells passed continuously for at least 6 months in culture. Tracks 1–5 contain *Bam*HI-cleaved whole cell DNA from lines 13/33 f4, f5 and f6, 31 f4L and 13/33 (uncloned), respectively, and tracks 6 and R contain cleaved DNA from control AGMK cells and EBV virions, respectively. In **c**, the probe was the nick-translated EBV fragment *Eco*RI H, carried on plasmid pAT153 (ref. 24), which hybridized to EBV DNA *Bam*HI fragments T, b and c, as shown (track R). **d**, The results obtained when the blot was washed at 68 °C overnight in low-salt conditions, then re-probed with nick-translated virion DNA and exposed for a longer time. The strongly hybridizing bands in tracks 1 and 2 (in **c**) are essentially no longer visible, whereas the weakly hybridizing bands (see tracks 3 and 5) remain. In addition, in **d**, a new band having a mobility intermediate between *Bam*HI co-migrating fragments B/C and D/E can be seen, especially in tracks 1 and 2. These data suggest that EBV DNA is present but that the strong bands seen in **c** cannot be exclusively correlated with viral DNA. A comparison of **c** and **d** with **a** and **b** further suggests that some change in sequence may have occurred during the passaging of the cells. Most notable is the apparent loss of the two most slowly migrating bands, with their possible replacement by a single, slowly migrating band in lines 13/33 f4 and f5 (tracks 1 and 2), and a faster migrating band in line 13/33 f6 and uncloned 13/33 cells (tracks 3 and 5). **e**, An experiment analogous to that described for **c** and **d**, but with 32 P-labelled nick-translated virion EBV DNA used as the initial probe. Two exposures are shown, and the major hybridizing bands are indicated by arrows. Whole cell DNA isolated from 13/33 f4 cells after more than a year in culture gave a result (not shown) indistinguishable from that found in track 1, indicating sequence stability and the continued presence of the viral DNA during cell propagation.

genome overcomes the senescence observed in untreated AGMK cells and confers the property of unlimited growth on the monkey epithelial cells in culture. Cells immortalized with the overlapping p13/p33 and p31 fragments, following propagation from isolated dense foci, had a finite capacity to grow in soft agar, however, and failed to produce tumours in whole animal (nude mice) experiments. Thus, it seems that of the multi-stage events necessary for the production of malignant cells¹⁵, not all have occurred in these epithelial cells. Note that normal human B lymphocytes, immortalized by EBV infection, initially also fail to produce tumours in nude mice¹⁶. The most obvious explanation for these data is that EBV *per se* lacks malignant capacity, and additional 'helper' functions, such as those derived from chemical carcinogens or human oncogenes, are necessary for inducing a final, transformed state of the cell. Our experiments have produced cell lines which should allow this conclusion to be explored *in vitro*.

The original choice of AGMK cells as an experimental system was based on the fact that expression of Epstein-Barr viral functions has been observed in many non-human primates^{17–19}

and that transformation efficiency, a possible reflection of stable integration of heterologous information, might be expected to be high²⁰. The main cautionary note to be considered in interpreting our data resides in the fact that an endogenous 'EBV-like' virus has been isolated from a lymphoblastoid African green monkey line (AGM-1106) by zur Hausen and colleagues²¹. While this lymphotropic virus would not be expected to be present in kidney cells, as EBV DNA has never been found in human kidney epithelial cells, it is conceivable that AGMK cells might already carry herpesvirus information that predisposes them to immortalization. The EBV sequences from the overlapping p13/p33 and p31 clones were unique, however, among the recombinant and other DNAs introduced during the experimentation protocol in producing immortalization (see Fig. 2). Competition experiments have shown that these sequences are absent from the original AGMK cells and that they survive in the established epithelial lines. Thus, they are undoubtedly important in the immortalization event.

Similar experiments are being done to study the interaction of fragments of EBV with other primate cells. Preliminary data

using primary kidney cells from common marmosets (*Callithrix jacchus*) and primary human breast cells (studies with N. Wedderburn and S. E. Chang) show that at least one of the EBV cloned DNA fragments (p31) which confers immortality on AGMK cells, also markedly promotes growth in these cells. As these studies are potentially relevant to the human disease, nasopharyngeal carcinoma, the experimental approach should be extended to a variety of human epithelial cells.

We thank Drs S. E. Chang and C. Rowlett for advice and help in showing the epithelial character of the cell lines described; Dr E. B. Lane for a generous gift of the monoclonal antibody, LE61; Dr D. Crawford and Professor G. Klein for assaying cells for EBNA expression; Ms E. Björck for assistance with molecular cloning; and A. Leigh-Brown for some of the initial cell work. We also thank Drs A. Harris and T. Lindahl for helpful discussions.

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- Epstein, M. A. *Adv. Cancer Res.* **13**, 383-411 (1970).
- Henle, W. & Henle, G. *Cancer Res.* **33**, 1419-1423 (1973).
- Klein, G. in *The Herpesviruses* (ed. Kaplan A. S.) 521-555 (Academic, New York, 1973).
- de Thé, G. in *Viral Oncology* (ed. Klein, G.) 769-797 (Raven, New York, 1980).
- Reedman, B. M. & Klein, G. *Int. J. Cancer* **50**, 307-314 (1973).
- Lindahl, T. *et al. Int. J. Cancer* **50**, 764-772 (1974).
- Hohn, B. & Collins, J. *Gene* **11**, 291-298 (1980).
- Griffin, B. E., Björck, E., Bjursell, G. & Lindahl, T. *J. Virol.* **40**, 11-19 (1981).
- Lane, E. B. *J. Cell Biol.* **92**, 665-673 (1982).
- Chang, S. E., Keen, J., Lane, E. B. & Taylor-Papadimitriou, J. *Cancer Res.* **42**, 2040-2053 (1983).
- Southern, E. M. *J. molec. Biol.* **98**, 503-517 (1975).
- Peden, K., Mounts, P. & Hayward, G. S. *Cell* **31**, 71-80 (1982).
- Arrand, J. R., Walsh-Arrand, J. E. & Rymo, L. *EMBO J.* **2**, 1673-1683 (1983).
- Summers, W. P. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 5688-5692 (1982).
- Farber, E. & Cameron, R. *Adv. Cancer Res.* **31**, 125-226 (1980).
- Nilsson, K., Giovanella, B. C., Stehlin, J. S. & Klein, G. *Int. J. Cancer* **19**, 337-344 (1977).
- Miller, G. *J. infect. Dis.* **130**, 187-205 (1974).
- Frank, A., Andiman, W. A. & Miller, G. *Adv. Cancer Res.* **23**, 171-201 (1976).
- Falk, L. *et al. Int. J. Cancer* **13**, 363-376 (1974).
- Gorman, C., Padmanabhan, R. & Howard, B. H. *Science* **221**, 551-553 (1983).
- Böcker, J. F., Tiedemann, K.-H., Bornkamm, G. W. & zur Hausen, H. *Virology* **101**, 291-295 (1980).
- Adams, A. in *Epstein-Barr Virus: Production, Concentration and Purification* (eds Ablashi, D. V., Aasestad, H. G. & de Thé, G.) 129-146 (Int. Agency Res. Cancer, Lyon, 1975).
- Hohn, B. *Meth. Enzym.* **68**, 299-309 (1979).
- Arrand, J. R. *et al. Nucleic Acids Res.* **9**, 2999-3014 (1981).
- Rigby, P. M., Dieckmann, M., Rhodes, C. & Berg, P. *J. molec. Biol.* **113**, 237-252 (1977).
- Dambaugh, T. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 2999-3003 (1980).
- Skare, J. & Strominger, J. L. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3860-3864 (1980).
- Van der Eb, A. S. & Graham, F. L. *Meth. Enzym.* **65**, 826-839 (1980).
- Lusky, M. & Botchan, M. *Nature* **293**, 79-81 (1981).

Initiation of translation at internal AUG codons in mammalian cells

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Initiation of translation of eukaryotic mRNAs typically occurs at the first AUG triplet from the 5' end of the message, although several notable exceptions have been described¹⁻³. Using vectors which efficiently express the gene encoding the surface antigen of hepatitis B virus in monkey cells, we have studied the consequences of inserting ATG triplets in all three reading frames upstream of the usual translational initiation codon of this gene. In agreement with the scanning model for eukaryotic translation initiation^{1,2}, these additional codons can severely depress the initiation of translation at the 'authentic' start codon, although the extent of inhibition depends on sequences flanking the upstream AUG. Such inhibition can, however, be at least partially suppressed by the presence of a translation termination codon in-frame with the upstream AUG. These results raise the possibility that mammalian ribosomes can reinitiate translation at an AUG codon after previously initiating, and terminating, at an upstream site.

We have used a rapid expression assay to quantify levels of the surface antigen of hepatitis B virus (HBsAg) production

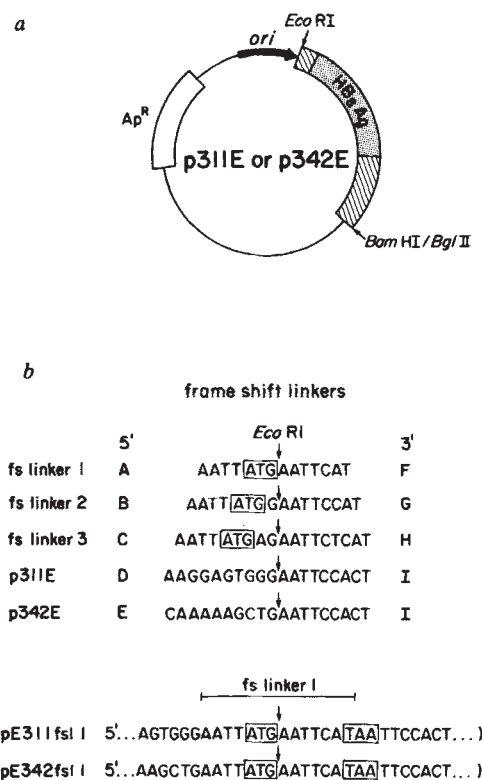


Fig. 1 Construction of pE311fsl and pE342fsl plasmids. The pE311fsl plasmids were derived from p311E (ref. 4), containing the 2,000-bp EcoRI-BglII fragment of hepatitis B virus (ref. 9) inserted into plasmid pML (ref. 18) between the EcoRI and BamHI sites and following the 311-bp EcoRII fragment of SV40 oriented in the early direction. This SV40 fragment spans the replication origin (nucleotides 5,092-160 (ref. 17)) and the early viral promoter⁵. The pE342fsl plasmids were derived from p342E (ref. 4), identical to p311E except that a 342-bp PvuII-HindIII fragment (nucleotides 5,171-270 (ref. 17)) spanning the SV40 origin was used instead of the 311-bp EcoRII fragment. Both plasmids were modified by incompletely digesting with EcoRI and filling-in the restriction site using DNA polymerase as described previously (ref. 19). Plasmids were screened for the loss of the EcoRI site preceding the SV40 early promoter. Into the single remaining EcoRI site immediately following the SV40 promoter were separately inserted the three oligonucleotides, synthesized by the phosphotriester method²⁰. These oligonucleotides are bounded by the self-complementary ends of the EcoRI site and contain an ATG triplet followed by an EcoRI site (b). The region spanning the EcoRI site used in the construction of every plasmid was directly sequenced using the dideoxynucleotide chain termination method²¹ to verify the structure of the linker and the reading frame. **a**, Schematic map of plasmids p311E and p342E. The HBsAg gene is denoted as a shaded box. Nontranslated regions are cross-hatched and the coding region is shown as a stippled box. The position of the SV40 origin/promoter fragment^{4,5} is shown. **b**, Sequence of the three oligonucleotide frameshift linkers. The sequences of the oligonucleotide linkers are shown as a 5' component (A, B, C) and a 3' component (F, G, H). D, E and I illustrate the sequence at the EcoRI site present at the junction of the SV40 and HBV inserts in p311E and p342E (ref. 4). The resulting sequence spanning the EcoRI site of pE311fsl-1 and pE342fsl-1 is shown.

directed by mutants of plasmids p342E and pE311 (ref. 4). These pBR322-based plasmids contain the gene encoding HBsAg, positioned behind either of two different, but overlapping, Simian virus 40 (SV40) DNA fragments which contain sequences spanning the viral origin of DNA replication and early promoter (ref. 5) (Fig. 1a). When introduced into COS cells (monkey cells which synthesize SV40 T antigen⁶), plasmid replication ensues with the concomitant synthesis of significant levels of HBsAg mRNA^{4,7}. We have previously demonstrated by S₁ nuclease analysis that such mRNA transcribed from both

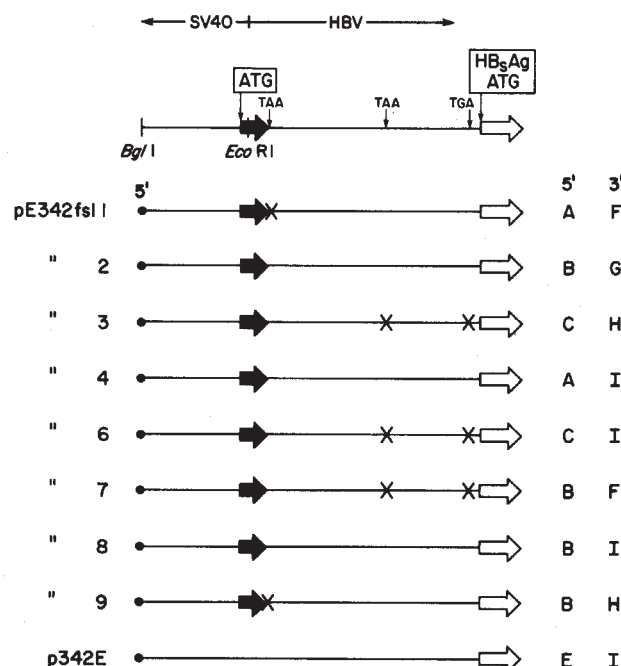


Fig. 2 Structure of the p342E frameshift plasmids. Plasmids p342E, pE342fsl-1, -2 and -3 were digested with *EcoRI* and *SalI*, the two resulting fragments separated and purified on an acrylamide gel using standard procedures¹⁹. The additional hybrid plasmids were constructed by ligation of the appropriate fragments. The structure of each was verified by direct determination of the nucleotide sequence across the restored *EcoRI* site²¹. The sequence of the SV40/hepatitis B virus junction is listed as a 5' and 3' component. The corresponding nucleotide sequence can be deduced from the information provided in Fig. 1b. X, The locations of termination codons in-frame with the linker ATG. The position of the linker ATG and the translation start codon of the HBsAg gene are denoted above the line drawing.

plasmids initiates within the SV40 promoter at the expected distance from the TATA box⁴ and terminates within HBV sequences 1,100 nucleotides 3' of the coding region of the HBsAg gene (ref. 7, and C.-C.L., C.C.S. and A.D.L., unpublished data). In pE342, the gene encoding the surface antigen is positioned directly 3' of SV40 sequences encompassing the complete early promoter⁴, such that no AUG triplets precede the translation initiation codon of HBsAg in the mRNA encoding HBsAg (ref. 4 and Fig. 1a). COS cells transfected with this plasmid synthesize and secrete significant quantities of HBsAg (ref. 4), a protein of 226 amino acids^{8,9} whose synthesis and secretion proceeds without the involvement of a cleavable signal peptide^{4,10,11}.

To assess directly the effects of upstream AUG triplets on downstream translational initiation, we inserted one of three synthetic *EcoRI* DNA linkers (fsl-1, -2 and -3, Fig. 1b), each containing an ATG triplet, into the unique *EcoRI* site of p342E (located at the junction of the SV40 and HBV sequences), to generate pE342fsl-1, pE342fsl-2 and pE342fsl-3. Note that insertion of each of these linkers introduces an upstream ATG triplet in a particular reading frame relative to the 'authentic' translational initiation codon of HBsAg (Fig. 2). These linkers contain the recognition sequence for the restriction endonuclease *EcoRI*; thus after insertion into the vector, a unique *EcoRI* site is restored. This facilitated the construction of hybrid vectors (pE342fsl-4, -6, -7, -8 and -9, Fig. 2), such that the effects of the reading frame of the upstream AUG triplet relative to the 'authentic' HBsAg initiation codon on HBsAg expression could be assessed independently of small sequence differences within the linker (for example, the nucleotide at position +4 relative to the 5' AUG (ref. 2)) which distinguish the three frameshift linkers (Fig. 1b). The effect of these insertions on

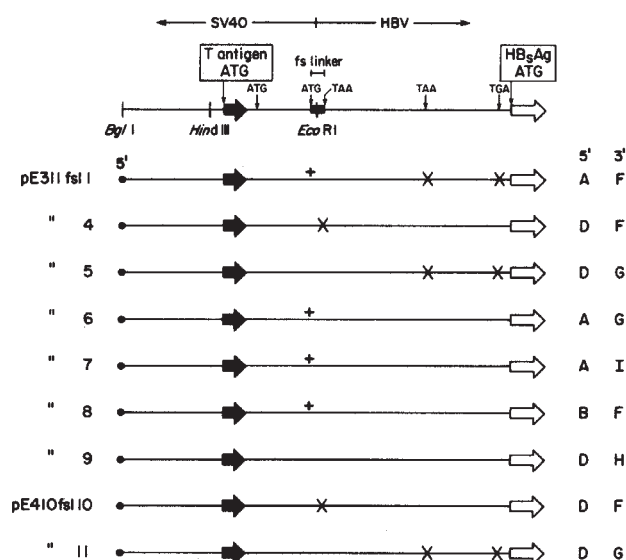


Fig. 3 Structure of the p311E frameshift plasmids. Plasmids p311E and pE311fsl-1, -2, and -3 were treated as described in Fig. 2 and the various combinations constructed as indicated. All junctions at the restored *EcoRI* site were verified by direct sequence analysis. Plasmids pE410fsl-10 and -11 were constructed by digesting p311E with *BglII* and *SalI*, and inserting the 2,400-bp fragment spanning the HBsAg gene between the equivalent sites of p342E (see also Table 2). Symbols are as described in Fig. 2; the presence of an ATG provided by the frameshift linker is indicated by a '+'. The location of the possible termination codons are also shown. The precise structure of the junction can be deduced from the sequences shown in Fig. 1b.

HBsAg expression was evaluated by transfecting COS cells with the various plasmids and assaying HBsAg in the medium 72 h later. Table 1 illustrates that many of the frameshift plasmids direct the synthesis of HBsAg at levels comparable to p342E, confirming that upstream AUG triplets do not necessarily impair internal initiation¹². These results extend previous descriptions of this phenomenon¹² since they include AUGs flanked by 'favourable' nucleotides^{2,3} at the -3 (A) and +4 position (G) (compare pE342fsl-7 and -9 with p342E). In contrast, plasmids pE342fsl-2, -4 and -8 direct the synthesis and secretion of somewhat lower amounts of HBsAg. As illustrated in Table 1, a common feature of these plasmids with a reduced ability to express HBsAg is the absence of an in-frame translational termination codon intervening between the AUG triplet within the synthetic linker and the AUG triplet defining the start of the HBsAg gene, suggesting that the presence of such a termination codon may enhance internal initiation at the HBsAg gene.

These results demonstrate that, although the presence of an upstream AUG triplet is not in itself sufficient to preclude translation initiation from an internal site (see also refs 13, 14), certain configurations of such a triplet can suppress internal initiation. To see whether flanking sequences could modulate the efficiency with which ribosomes recognize AUG codons as initiation sites^{2,3}, we examined the effects of inserting an 'authentic' translation initiation codon 5' of the gene encoding HBsAg, using plasmid p311E (ref. 4), which, although similar to pE342, contains additional sequences 3' of the SV40 early promoter, including the translational initiation site for SV40 T antigen. As we have reported, plasmid p311E is severely restricted in its ability to promote the synthesis and secretion of HBsAg synthesis⁴. Previous evidence indicated that this block is not a consequence of transcriptional impairment resulting from the absence of the enhancer element associated with the 72-base pair (bp) repeat unit within the SV40 promoter⁴, suggesting that HBsAg is translated very poorly, if at all, from its message. This was confirmed by examining HBsAg synthesis from additional plasmid constructions (p232E and p410E). Plasmid

Table 1 Expression of pE342fsl plasmids in COS cells

Plasmid	Linker* sequence		Reading† frame	Termination‡ codon	HBsAg§
	5'	3'			
pE342fsl-1	A	F	+	+	17,145 ± 1,925
pE342fsl-2	B	G	same	—	9,175 ± 20**
pE342fsl-3	C	H	-1	+	23,620 ± 1,640
pE342fsl-4	A	I	same	—	6,235 ± 1,235
pE342fsl-6	C	I	-1	+	17,440 ± 1,260
pE342fsl-7	B	F	-1	+	19,645 ± 550**
pE342fsl-8	B	I	+1	—	1,980 ± 280
pE342fsl-9	B	H	+1	+	22,580 ± 580
p342E	E	I	—	—	25,785 ± 5,560
p232E	E	I	—	—	16,000 ± 600**

Confluent 60-mm dishes of COS-1 cells⁶ were transfected in duplicate with 0.5 µg plasmid DNA using a modified DEAE-dextran method¹⁶ as described elsewhere⁴. After 16 h, the cells were washed once with Dulbecco's modified Eagle's medium (DMEM), then fed with 3 ml 7% calf serum in DMEM. Two days later, the cells were re-fed with fresh media. After 24 h, the amount of HBsAg secreted into the medium was quantitated by RIA using the Ausria II kit (Abbott).

* Sequence of frameshift linker as described in Fig. 1 legend.

† Reading frame of linker ATG relative to that of the HBsAg.

‡ Presence (+) or absence (—) of a termination codon in frame with the upstream ATG.

§ Counts per minute as assayed by Ausria II kit, corrected for included negative control. Results are averaged values (with ranges given) obtained from four (**) or two) independent experiments.

|| p232E was constructed by exchanging the proximal promoter sequences of p311 (which lack the SV40 72-bp repeats) with those of pE342 (which lack SV40 T antigen coding information) at the *Bgl*I site. p232E thus contains neither an intact 72-bp repeat nor the translational initiation codon of SV40 T antigen (SV40 sequences extending from the *Eco*RI site at position 160 to the *Hind*III site at position 5,171 (ref. 17)).

Table 2 HBsAg expression of pE311fsl plasmids in COS cells

Plasmid	Linker* sequence		Reading† frame	Termination‡ codon	HBsAg*
	5'	3'			
p311E	D	I	same	—	60 ± 50
pE311fsl-1	A	F	-1	+	1,290 ± 205
pE311fsl-4	D	F	+1	+	2,725 ± 310
pE311fsl-5	D	G	-1	+	1,350 ± 155
pE311fsl-6	A	G	same	—	90 ± 45
pE311fsl-7	A	I	+1	—	460 ± 35
pE311fsl-8	B	F	same	—	20 ± 25
pE311fsl-9	D	H	same	—	125 ± 10**
p410E§	D	I	same	—	30 ± 30
pE410fsl-10	D	F	+1	+	2,895 ± 105**
pE410fsl-11	D	G	-1	+	2,130 ± 180**

Methods of transfection and assay are as described in the legend to Table 1.

* Headings are as defined in Table 1.

† Reading frame of T-antigen translation initiation codon relative to that of HBsAg initiation codon.

‡ p410E was created by the exchange of the proximal SV40 promoter sequences of p342E with the distal promoter sequences of p311E using the SV40 *Bgl*I site. p410E thus contains both SV40 72-bp repeats as well as the translational initiation codon of SV40 T antigen (SV40 sequences from the *Pvu*II site at position 270 to the *Eco*RII site at position 5,092 (ref. 17)).

p232E lacks sequences comprising the 72-bp repeat, but is otherwise identical to pE342. p232E and p342E produce similar levels of HBsAg (Table 1) consistent with the interpretation that the HBV genome contains enhancer sequences capable of complementing those of SV40 (ref. 4), while the insertion of the SV40 enhancer sequences into p311E (generating p410E) fails to restore HBsAg synthesis (Table 2). Thus the inability of p311E to promote efficient HBsAg synthesis appears to result from an extreme inhibitory effect of the SV40 T-antigen translation initiation codon located 231 nucleotides 5' of the AUG codon which normally initiates HBsAg synthesis.

Protein synthesis initiating at the first AUG (that of T antigen) from mRNA directed by p311E would be expected to produce a hybrid polypeptide consisting of the first 24 amino acids of SV40 T antigen, 52 amino acids specified by the sequence immediately preceding the HBsAg gene, and 226 amino acids of HBsAg (ref. 4). Consistent with this interpretation, intracel-

lular extracts of COS cells transfected with p311E do contain an extended form of HBsAg as determined by radioimmunoassay (RIA) and immunoprecipitation (data not shown), indicating that a polypeptide with HBsAg determinants is synthesized in a form which cannot be secreted. To examine this phenomenon in more detail, the frameshift linkers were incorporated into the unique *Eco*RI site of the parent plasmid p311E or p410E (Fig. 1), and pertinent hybrid plasmids were prepared (Fig. 3). The ability of the various constructs to promote HBsAg synthesis was determined by assaying the medium of transfected COS cells 72 h after DNA administration.

Table 2 illustrates that, as with pE311, no secreted HBsAg is observed with any frameshift linker plasmid expected to generate a fused T antigen/HBsAg polypeptide. In marked contrast, however, significant levels of HBsAg are synthesized from those plasmids which contain an in-frame translational termination codon following the translational initiation codon of SV40 T antigen. This effect was independent of the presence of an AUG triplet within the linker sequence (compare pE311fsl-1 with pE311fsl-5). Specifically, consider that plasmids pE311fsl-6, -8 and -9 all produce less than 1% control levels (that produced by p232E) of HBsAg. These three plasmids are not identical, yet in each the 5' AUG is present in the same reading frame as the surface antigen AUG, with no intervening in-frame termination codons. Plasmid pE311fsl-7 produces less than 3% of the control level of HBsAg; like the others it lacks an in-phase termination codon, but differs by having the SV40 T antigen initiator AUG in the second reading frame relative to the surface antigen AUG. In contrast, those plasmids (both p311E and p410E-based) in which an in-frame termination codon intervenes between the 5' AUG and the AUG encoding the first amino acid of HBsAg direct the synthesis of significantly more HBsAg. Interestingly, the presence of an in-frame translational termination codon following the upstream AUG does not restore levels of HBsAg produced by the pE311 vectors to those produced by p342E or p232E (compare Tables 1 and 2). This could be due to the inability of a termination codon to promote the highly efficient use of a downstream initiation codon; alternatively the structure of the hybrid T antigen/HBsAg mRNA may preclude efficient translation of the HBsAg gene.

It has previously been proposed that nucleotides in positions -3 (A) and +4 (G) facilitate recognition of the AUG codon by eukaryotic ribosomes². Our results suggest that additional sequences may be involved in this process, since internal initiation occurs much less frequently when an 'authentic' AUG is positioned upstream of the HBsAg gene (pE311 and pE410-based vectors) than when synthetic AUGs conforming to these specifications are present upstream (pE342-based vectors). The nature of such sequences is uncertain, but the possibility remains that a 'functional' equivalent of a ribosome binding site exists, despite evidence that most mRNAs do not reveal any indication of a specific base-pairing ability between their 5' non-coding segments and the 3' terminus of 18S rRNA (ref. 15).

The fact that internal initiation at an otherwise suitable AUG triplet can be thoroughly suppressed by an upstream AUG codon lends strong support to the scanning model for eukaryotic translation initiation^{1,2}, and is inconsistent with the concept that ribosomes bind directly to AUG initiator codons in mRNA irrespective of their proximity to the 5' terminus¹². In contrast to previous suggestions that internal initiation can occur only when upstream AUG triplets are flanked by unfavourable nucleotides², our results suggest that reinitiation of translation can occur after the efficient utilization of upstream AUG codons, provided translation has been terminated before the ribosome has reached the internal AUG. It is unclear what factors dictate the efficiency with which such reinitiation occurs; this may be governed to some extent by the position of the termination codon relative to the flanking AUG codons. The fact that internal initiation does not occur in the absence of such a termination codon in the pE311-based vectors strongly argues that initiation at the internal AUG triplet (in those vectors

containing an intervening termination codon) is not mediated by ribosomes which 'escaped' initiation at the upstream site², but rather by true reinitiation, although we stress that in a general sense the two models are not incompatible with one another. For example, internal initiation at a site preceded by an efficiently utilized AUG codon may proceed only when translation from the upstream site has first terminated. However, in those instances where only inefficiently recognized upstream AUG triplets are present, an in-frame termination codon would not be required for efficient translation at the internal AUG, since migrating 40S ribosome subunits may simply bypass a weak AUG. In view of the observation that in-frame termination

codons almost always follow upstream AUGs in those messages where translation initiates internally, it has been proposed that such terminators may function to facilitate the early return to circulation of those ribosomes which initiate at the upstream site². The prospect that ribosomes may not be released immediately after translation termination, but rather continue scanning for at least some distance, provides an additional rationalization for this observation, and suggests a mechanism by which polycistronic transcripts may function in mammalian cells.

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1. Kozak, M. *Curr. Topics Microbiol. Immun.* **93**, 81-123 (1981).
2. Kozak, M. *Nucleic Acids Res.* **9**, 5233-5253 (1981).
3. Kozak, M. *Microbiol. Rev.* **47**, 1-45 (1983).
4. Crowley, C., Liu, C. C. & Levinson, A. D. *Molec. cell. Biol.* **3**, 44-55 (1983).
5. Benoist, C. & Chambon, P. *Nature* **290**, 304-310 (1981).
6. Gluzman, Y. *Cell* **23**, 175-182 (1981).
7. Simonsen, C. C. & Levinson, A. D. *Molec. cell. Biol.* **3**, 2250-2258 (1983).
8. Valenzuela, P. *et al. Nature* **280**, 815-819 (1979).
9. Valenzuela, P., Quiroga, J., Zaldivar, P., Gray, P. & Rutter, W. J. in *Animal Virus Genetics* (eds Fields, B., Jaenisch, R. & Fox, C. F.) (Academic, New York, 1980).
10. Moriarty, A. M., Hoyer, B. H., Shih, J. W., Gerin, J. L. & Hamer, D. H. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2606-2610 (1981).
11. Liu, C. C., Yansura, D. & Levinson, A. D. *DNA* **1**, 213-223 (1982).
12. Lomedico, P. T. & McAndrew, S. J. *Nature* **299**, 221-225 (1982).
13. Jay, G. *et al. Nature* **291**, 346-349 (1982).
14. Mulligan, R. C. & Berg, P. *Molec. cell. Biol.* **1**, 449-459 (1981).
15. De Wachter, R. *Nucleic Acids Res.* **7**, 2045-2054 (1979).
16. Sompayrac, L. M. & Danna, K. J. *Proc. natn. Acad. Sci. U.S.A.* **78**, 7575-7578 (1981).
17. Toozé, J. *DNA Tumor Viruses: Molecular Biology of Tumor Viruses*, Pt 2 (Cold Spring Harbor, New York, 1981).
18. Lusk, M. & Botchan, M. *Nature* **293**, 79-81 (1981).
19. Gray, P. W. *et al. Nature* **295**, 503-508 (1982).
20. Crea, R., Kraszewski, A., Hirose, T. & Itakura, K. *Proc. natn. Acad. Sci. U.S.A.* **75**, 5765-5769 (1978).
21. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463-5467 (1977).

Nucleotide sequence of avian retroviral oncogene *v-mil*: homologue of murine retroviral oncogene *v-raf*

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Eukaryotic cells contain genes termed proto-oncogenes (*c-onc*) which have the potential to transform cells in culture and induce tumours *in vivo*. Most of these genes have been identified by their occasional incorporation into retroviral genomes which can act as natural transducing vectors for these and perhaps other cellular genes^{1,2}. Cell-derived oncogenes of retroviruses (*v-onc*) are associated mostly with the induction of mesenchymal tumours whereas carcinoma induction is rare³. One of these rare carcinoma-inducing viruses is the acutely transforming avian retrovirus MH2 (refs 3-5). Recently we^{6,7} and others⁸ have shown that this virus carries a novel putative oncogene, *v-mil*, in addition to the known oncogene *v-myc*. While the transforming ability of *v-mil* has not been directly established, we have recently discovered by hybridization analysis that *v-mil* is homologous to *v-raf* (ref. 9), the transforming gene of the murine retrovirus 3611 MSV (ref. 10). Both viruses express the *mil/raf* oncogene product as a *gag*-fusion polypeptide^{6,11}, while the *myc* oncogene of MH2 is expressed via a subgenomic mRNA¹². Here we report the complete nucleotide sequence of *v-mil* and compare it with that of *v-raf*^{3,4}. The 80% homology between the nucleotide sequences and the 94% homology between the predicted amino acid sequences of the two viral genes clearly indicate that these are the avian and murine forms of the same gene. Comparison of the two sequences with that of the human cellular homologue¹³ (T. I. Bonner *et al.*, manuscript in preparation) indicates that *v-raf* has more 3' untranslated sequences while *v-mil* has additional sequences from two 5' exons of the cellular homologue. Although the *mil/raf* amino acid sequences reveal partial homology to that of the *v-src* product, no tyrosine-specific protein kinase activity is detected for the *gag-mil* and the *gag-raf* hybrid proteins.

1,416 nucleotides of MH2 proviral sequence containing the complete *v-mil* sequence as well as adjacent viral *gag* p12 sequence at its 5' end and *myc* gene sequence at its 3' end were determined and compared with the corresponding *v-raf* sequence in Fig. 1. The 5' end of *v-mil* is tentatively located at nucleotide 93 or 94. This determination is based on the observation that nucleotides 1-92 are homologous to the *gag* p12 sequence of Rous sarcoma virus (RSV)¹⁴ while the following nucleotides are not. In addition, comparison with the human homologue *c-raf-1* shows homology beginning at nucleotide 94. Nucleotides 94-265 are specific to *v-mil*. Comparison with the human cellular homologue indicates that they are derived from three exons, one of which is exon 1 of the chicken cellular clone *c-mil-4* (refs 7, 15) and two of which were not identified in the chicken clone. Homology to the *v-raf* sequence begins at position 266 and with the exception of two small insertions or deletions continues to the termination codon at nucleotide 1,233. The *v-mil* sequence ends 12 nucleotides 3' of the termination codon. Beyond this point, the sequence matches that of the *myc* cellular homologue, beginning in the first intron 172 nucleotides 5' of the second exon (M. Linial, personal communication). The *v-mil* sequence contains an insertion of a nucleotide at 1,209 coupled with the deletion of four nucleotides between 1,215 and 1,218 which preserves the reading frame to the termination codon. The human homologue shows no deletions in this region relative to *v-raf*. Thus, the insertions occurred in the mammalian lineage before the divergence of humans and mice or deletions arose in the chicken lineage. Alternatively, the deletions may have arisen during viral replication of MH2 after its acquisition of the *v-mil* sequence. The nucleotide sequence homology over this common coding region is 80% with most of the changes occurring as silent third position changes so that the predicted amino acid sequences have 94% homology.

The MH2 sequence has only one open reading frame capable of producing the *gag-mil* polypeptide of molecular weight 100,000. This reading frame corresponds to the reading frame used to produce the *gag-raf* polypeptide. Assuming that the *gag* sequence of MH2 is the same as that of RSV (ref. 14), the predicted polypeptide would contain 514 *gag*-specific amino acids and 380 *mil*-specific amino acids with a calculated molecular weight of 97,014. This agrees well with the molecular weight of the *gag-mil* p100 protein determined by SDS-polyacrylamide gel electrophoresis, considering the fact that the protein is phosphorylated and possibly glycosylated. There are three potential glycosylation sites of the form Asn-X-(Thr or Ser) in the *mil* sequence at amino acid positions 16-18, 32-34 and 350-352. Of the 19 amino acid differences between *v-mil*

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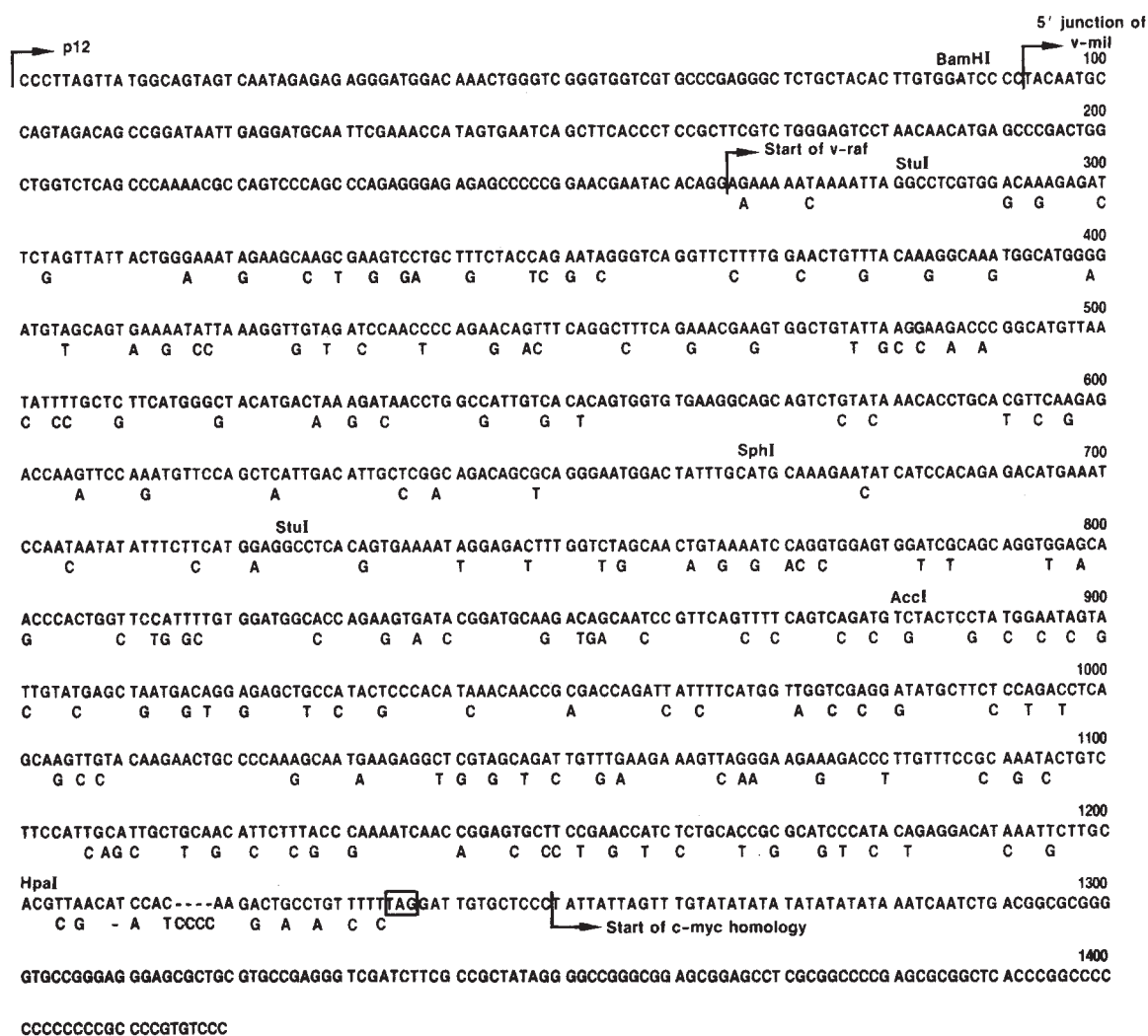


Fig. 1 Nucleotide sequence comparison between the *mil* region of MH2 proviral DNA and *v-raf*³⁴. The complete *v-mil* sequence is shown with partial sequences of adjacent viral *gag* and *v-myc* genes. The *v-raf* coding sequence is shown below the *v-mil* sequence at nucleotides where it differs from *v-mil*, and deleted nucleotides are indicated by dashes. The 5' borders of *v-mil* and *v-raf* are indicated by arrows. The termination codon common to both sequences is boxed. The location of the *mil-myc* junction is indicated by an arrow. Restriction sites used for sequencing of MH2 DNA by the Maxam-Gilbert method³⁵ are indicated above the nucleotide sequence.

and *v-raf* (Fig. 2), one occurs at a position where *v-mil*, *v-raf* and human *c-raf-1* differ (T.I.B. *et al.*, manuscript in preparation), 11 occur at a position where *v-raf* and *c-raf-1* are the same and six occur at a position where *v-mil* and *c-raf-1* are the same. This result is consistent with mice being more closely related to humans than chickens.

A computer search of known protein sequences revealed that the *mil/raf* oncogene is related to members of the *src* family of oncogenes (see below). The homology is to the carboxy half of the p60 *v-src* product which is associated with tyrosine-specific protein kinase activity. Since the *v-raf* product has not shown any tyrosine kinase activity¹¹, it was unexpected that this homology exists for the *mil/raf* oncogene. The fact that the *v-mil* protein contains 59 more amino acids than *v-raf* made it important to test the *v-mil* product for kinase activity in comparison with the *raf* protein and with a known tyrosine-specific kinase specified by a member of the *src* family of oncogenes. Figure 3 compares protein kinase activity associated with proteins immunoprecipitated from cells transformed by MH2, 3611 MSV or avian Fujinami sarcoma virus (FSV) carrying the *fps* oncogene^{1,2}. Quail embryo cells or rat cells transformed by FSV contain the p140 *gag-fps* protein which is phosphorylated and associated with tyrosine-specific protein kinase activity¹⁶⁻¹⁸ leading to efficient *in vitro* phosphorylation of the p140 protein in a kinase assay (Fig. 3, lanes 1, 3). In the same conditions,

very low levels of phosphorus are transferred to the p75 *gag-raf* protein of 3611 MSV or the p100 *gag-mil* protein of MH2 (Fig. 3, lanes 5, 7). Furthermore, phosphoamino acid analysis revealed that this phosphorylation occurred at threonine and serine residues but not at tyrosine residues as in the *gag-fps* protein (data not shown). This agrees with the phosphorylation pattern observed *in vivo* for these proteins^{11,17,19}. Whether the low level of *in vitro* phosphorylation of the *raf* and *mil* proteins is an intrinsic activity or due to cellular enzymes present in the immunoprecipitates remains to be determined.

There are three other oncogenes, *v-erb* (ref. 20), *v-fms* (ref. 21) and *v-mos* (ref. 22), which are apparently negative for protein kinase activity yet show homology to the kinase portion of *v-src*. Their cellular locations suggest some diversity of function. The product of *v-erb-B* has been shown to be a membrane glycoprotein²³, possibly functioning as a growth factor receptor²⁴, whereas *v-fms* encodes a glycoprotein associated with the membranes of endoplasmic reticulum²¹. In contrast, fluorescence assays indicate a diffuse cytoplasmic distribution of the *v-mil*²⁵ and *v-mos*²⁶ proteins with *v-mil* possibly being associated with RNA²⁵. To determine any similarities among these proteins which may distinguish them from the kinase-positive *src*-related genes, we have compared their amino acid sequences in Fig. 4. Four regions show extensive homology, that is amino acids 18-50, 120-138, 157-183 and 202-247.



Fig. 2 The predicted amino acid sequence encoded by *v-mil* compared with that of *v-raf*. The amino acids are numbered beginning with the first *v-mil* specific amino acid. The *v-raf* sequence is shown below *v-mil* where it differs from *v-mil*, and deletions are indicated by dashes.

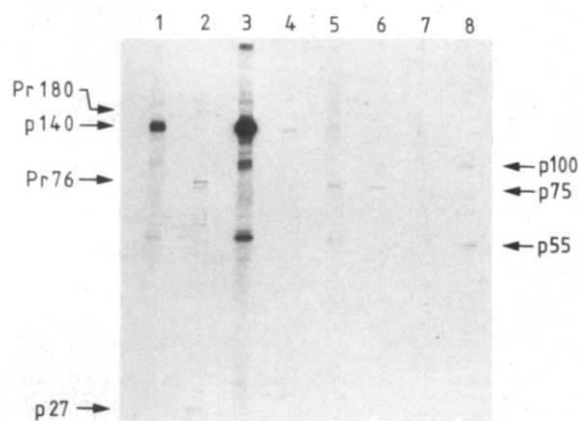


Fig. 3 Protein kinase activity in immunoprecipitates from lysates of cells transformed by FSV, 3611 MSV or MH2. Quail embryo cells transformed by FSV (FAV) (lanes 1, 2), FSV-transformed rat 3Y1 cells¹⁸ (lanes 5, 6), and a nonproducer quail cell clone transformed by MH2 (lanes 7, 8) were used. Lysates were prepared as described previously¹⁶ from unlabelled and ³⁵S-methionine-labelled cells using a lysis buffer containing 50 mM NaCl, 25 mM Tris-HCl pH 7.4, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA and 400 units ml⁻¹ Antagasan (Behringwerke) as a protease inhibitor. Cell lysates were preabsorbed with preimmune rabbit or goat sera and protein A-Sepharose, and immunoprecipitates were then prepared with rabbit antiserum against avian *gag* proteins⁶ (lanes 1, 2, 3, 4, 7 and 8) or with goat antiserum against the murine p15 *gag* protein¹¹ (lanes 5, 6) followed by absorption to protein A-Sepharose. Unlabelled immunoprecipitates were washed with lysis buffer and 20 mM Tris-HCl pH 7.4 and were then incubated for 15 min at 30 °C in 50 µl of a kinase reaction solution containing 20 mM Tris-HCl pH 7.4 10 mM MgCl₂, 1 mM dithiothreitol and 5 µCi [γ -³²P]ATP (~2,000 Ci mmol⁻¹; Amersham Buchler, Braunschweig). Reaction products were analysed on a 12.5% polyacrylamide-SDS gel (lanes 1, 3, 5, 7). Immunoprecipitates of ³⁵S-methionine-labelled proteins were analysed in parallel (lanes 2, 4, 6, 8). They include the p140 *gag-fps* protein of FSV (lanes 2, 4), the structural proteins Pr180, Pr76 and p27 of the helper virus FAV (lane 2), the p75 *gag-raf* protein of 3611 MSV (lane 6), and the p100 *gag-mil* protein of MH2 and a possible processing product p55 (lane 8). The autoradiograph was exposed for 12 h.

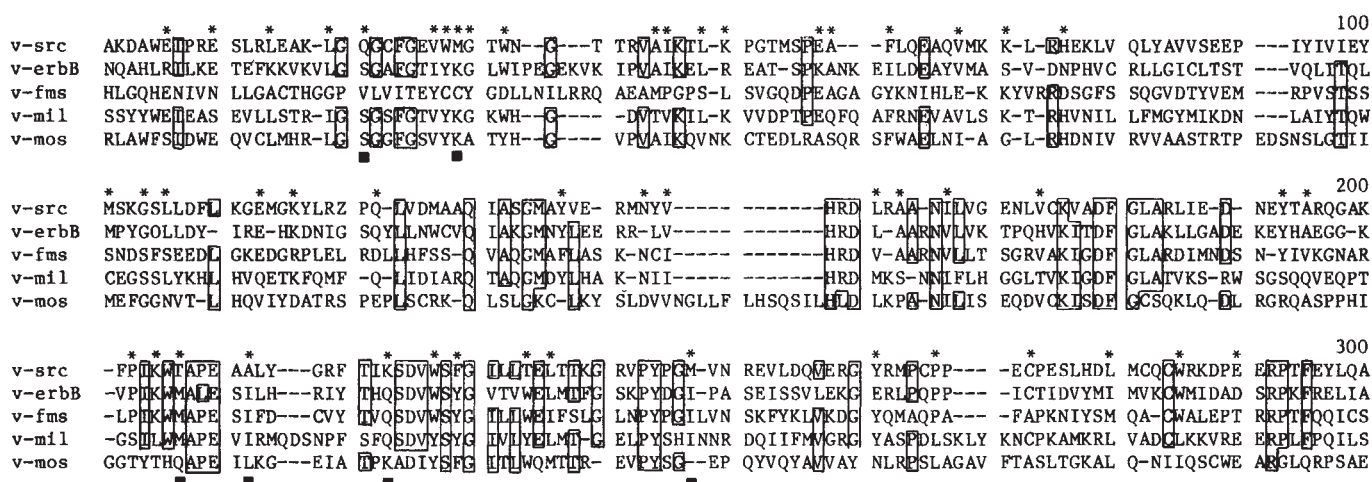


Fig. 4 Alignment of amino acids encoded by non-kinase oncogenes with those encoded by the *v-src* oncogene. The alignment begins at amino acid 71 of *v-mil* and continues to 337. No extensive homology was found outside these limits. Positions at which four or five of the sequences match are boxed. Positions at which three sequences match are indicated by an asterisk above the *v-src* sequence. Positions at which three of the four non-kinase genes match and are different from the kinase positive oncogenes (*fgr*, *yes*, *fes*, *fms*, *abl* and *src*) are indicated by a closed box below the *v-mos* sequence. The phosphotyrosine of *v-src* occurs at position 193. A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

Although the other four proteins show substantial homology in the first region, the *fms* sequence shows almost no homology before positions 100–120. The remaining kinase negative proteins (as well as the *raf* protein) contain the sequence Gly-Ser-Gly-X-Phe-Gly-X-X-X-Tyr at positions 21–29 with the serine and tyrosine residues distinguishing them from the kinase-positive oncogenes *src*, *fgr*, *yes*, *fms*, *fes* and *abl* (refs 14, 27–31). It has been suggested that the Gly-X-Gly-X-Gly sequence represents a nucleotide-binding site³². A similar sequence, Leu-Gly-Thr-Gly-Phe-Gly-Lys-Val-Val-Glu, is found in *v-fms* about 140 amino acids further towards its amino terminal. The remaining four positions where a common amino acid distinguishes the non-kinase genes, except for *mos*, from the kinase-positive genes occur in the fourth homology region. This region is functionally important as mutagenesis of the alanine at position 211 of *v-src* inactivates its kinase activity³³.

There are three known examples of a retrovirus containing what appear to be two oncogenes: AEV containing *erbA* and *erbB* (ref. 36), E26 containing *ets* and *myb* (refs 37, 38), and MH2 containing *mil* and *myc* (refs 5–7). In none of these cases have both genes been demonstrated to be transforming. The present data demonstrate that *v-mil* has 94% amino acid homology to *v-raf*. When this result is compared with the 96% amino acid homology of *v-raf* to its human homologue *c-raf-1*, it is clear that *mil* is the avian homologue of the murine *raf* gene. As both *v-raf* and the human homologue *c-raf-1* are capable of transformation, we infer that *mil* is also.

Note added in proof: Close homology has been reported³⁹ between the *mht* (*mil*) sequence of MH2 and our *v-raf* sequence. The reported *mht* sequence is nearly identical to our *mil* sequence; however, the authors identify the *c-myc* intron sequence as being *mht* 3' untranslated sequence.

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- Bishop, J. M. A. *Rev. Biochem.* **52**, 301–354 (1983).
- Bister, K. & Duesberg, P. H. in *Advances in Viral Oncology* Vol. 1 (ed. Klein, G.) 3–42 (Raven, New York, 1982).
- Beard, J. W. in *Viral Oncology* (ed. Klein, G.) 55–87 (Raven, New York, 1980).
- Carr, J. G. *Br. J. Cancer* **14**, 77–82 (1980).
- Alexander, R. W., Moscovici, C. & Vogt, P. K. *J. natn. Cancer Inst.* **62**, 359–366 (1979).
- Jansen, H. W., Patschinsky, T. & Bister, K. *J. Virol.* **48**, 61–73 (1983).
- Jansen, H. W., Rückert, B., Lurz, R. & Bister, K. *EMBO J.* **2**, 1969–1975 (1983).
- Kan, N. C. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 6566–6570 (1983).
- Jansen, H. W. *et al. Nature* **307**, 281–284 (1984).
- Rapp, U. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 4218–4222 (1983).
- Rapp, U. R., Reynolds, F. H. & Stephenson, J. R. *J. Virol.* **45**, 914–924 (1983).
- Pachl, C., Biegalka, B. & Linial, M. *J. Virol.* **45**, 133–139 (1983).
- Bonner, T. I. *et al. Science* **223**, 71–74 (1984).
- Schwartz, D. E., Tizard, R. & Gilbert, W. *Cell* **32**, 853–869 (1983).
- Bister, K. *et al. UCLA Symp. molec. cell. Biol.* **17** (in the press).
- Bister, K., Lee, W.-H. & Duesberg, P. H. *J. Virol.* **36**, 617–621 (1980).
- Feldman, R. A., Hanafusa, T. & Hanafusa, H. *Cell* **22**, 757–765 (1980).
- Mathey-Prevot, B., Hanafusa, H. & Kawai, S. *Cell* **28**, 897–906 (1982).
- Ramsay, G., Hayman, M. J. & Bister, K. *EMBO J.* **1**, 1111–1116 (1982).
- Yamamoto, Y. *et al. Cell* **35**, 71–78 (1983).

- Hampe A., Gobet, M., Sherr, C. J. & Galibert, F. *Proc. natn. Acad. Sci. U.S.A.* **81**, 85–89 (1984).
- van Beveren, C. *et al. Nature* **289**, 258–262 (1981).
- Hayman, M. J. *et al. Cell* **32**, 579–588 (1983).
- Downward, J. *et al. Nature* **307**, 521–527 (1984).
- Bunte, T., Greiser-Wilke, I. & Moelling, K. *EMBO J.* **2**, 1087–1092 (1983).
- Papko, J., Nigg, E. A. & Hunter, T. *Cell* **33**, 161–172 (1983).
- Naharro, G., Robbins, K. C. & Reddy, E. P. *Science* **223**, 63–66 (1984).
- Kitamura, N., Kitamura, A., Toyoshima, K., Hirayama, Y. & Yoshida, M. *Nature* **297**, 205–208 (1982).
- Shibuya, M. & Hanafusa, H. *Cell* **30**, 787–795 (1982).
- Hampe, A., Laprevotte, I., Galibert, F., Fedele, L. A. & Sherr, C. J. *Cell* **30**, 775–785 (1982).
- Reddy, E. P., Smith, M. J. & Srinivasan, A. *Proc. natn. Acad. Sci. U.S.A.* **80**, 3623–3627 (1983).
- Wierenga, R. K. & Hol, W. G. J. *Nature* **302**, 842–844 (1983).
- Bryant, D. & Parsons, T. *J. Virol.* **45**, 1211–1216 (1983).
- Mark, G. E. & Rapp, U. R. *Science* (submitted).
- Maxam, A. M. & Gilbert, W. *Meth. Enzym.* **65**, 499–560 (1980).
- Frykberg, L. *et al. Cell* **32**, 227–238 (1983).
- Nunn, M. F., Seeburg, P. H., Moscovici, C. & Duesberg, P. H. *Nature* **306**, 391–395 (1983).
- Leprince, D. *et al. Nature* **306**, 395–397 (1983).
- Kan, N. C. *et al. Science* **223**, 813–816 (1984).

Seismological evidence for shallow crystalline basement in Southern Uplands, Scotland

HALL *et al.*¹ propose that the Southern Uplands contain crystalline rocks of continental affinity at shallow depths ($\sim 1\text{--}5$ km). We suggest that the same seismic data can, in fact, be used to strengthen the argument for a thick accretionary prism².

It has been inferred that prehnite-pumpellyite facies metamorphism in the Southern Uplands is related to tectonic burial and rotation of the various slices of the prism as they were accreted during closure of the Iapetus Ocean, and that isograds in the prism are now subparallel to the present day erosion surface³⁻⁶. The colour alteration of conodonts in the Northern Belt indicates temperatures between 300 °C and 400 °C (refs 7, 8). The transition of the prehnite-pumpellyite to the greenschist facies lies between 300 °C and 350 °C (ref. 9), thus it has been suggested^{4,5} that greenschist facies is present not far below the present day surface over much of the Southern Uplands.

The LISPB seismic profile for the Grampian sector shows P-wave velocities similar to the Southern Uplands Seismic Profile (SUSP¹, that is, 6 km s^{-1} at the surface increasing to 6.05 km s^{-1} at 12 km depth¹⁰. Mainly amphibolite facies sediments (and granite) occur in the northern part and foliated greenschist facies greywackes occur in the south¹¹. In New Zealand, the seismic profile for the Otago greenschist terrane shows a velocity of 6 km s^{-1} for the depth range 1–10 km, lying above a velocity of 6.7 km s^{-1} (ref. 12). In Otago the transition from prehnite-pumpellyite facies to greenschist facies is marked by a transition of poorly foliated greywackes to strongly foliated quartzo-feldspathic schists¹³. Individual mica- and chlorite-schists from Vermont, USA, have experimentally determined velocities (at the appropriate pressures) similar to SUSP¹⁴. We conclude that the SUSP velocities are compatible with the occurrence of foliated greenschist facies metagreywackes at depths 1–5 km below the present surface.

Velocity anisotropism in foliated metamorphic rocks is well known^{12,15} and the 15% anisotropy measured at Eskdalemuir (EKA)¹ is very similar to that in slate, chlorite-schist and mica-schist measured experimentally^{14,16} at the appropriate pressure. Similar anisotropism is present at the Broughton array (BTN)¹ if the contribution of arrivals from the Midland Valley is ignored. We conclude that the seismic evidence from the BTN and EKA arrays supports the existence of sub-vertical, NE-SW foliated greenschists at a shallow depth.

Hall *et al.*¹ reinterpreted the Southern Uplands LISPB sector¹⁰ in terms of block-faulting of crystalline crust. We are not competent to quantify a seismic model involving the block-faulting of a thin layer of isotropic greywackes lying above anisotropic greenschists but we point out that the boundaries between the seismic blocks¹ correspond well with existing block faults⁵.

The geological arguments of Hall *et al.*¹ are equivocal: the xenoliths could originate from English continental crust subducted below the Southern Uplands²; the Midland Valley conglomerates may have come from the upper 11–17 km of the prism (Cockburnland¹⁷) which has since been stripped off⁴.

We conclude that the seismic evidence supports a thick accretionary prism in the Southern Uplands. We predict that a suitably positioned borehole sited in blocks F2 or F3 will penetrate greenschist facies metagreywackes rather than crystalline continental basement.

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- Hall, J. *et al.* *Nature* **305**, 418–420 (1983).
- Leggett, J. K., McKerrrow, W. S. & Soper, N. J. *Tectonics* **2**, 187–210 (1983).
- Oliver, G. J. H. *Nature* **274**, 242–243 (1978).
- Oliver, G. J. H. & Leggett, J. K. *Trans. R. Soc. Edinb.* **71**, 235–246 (1980).
- Leggett, J. K., McKerrrow, W. S. & Eales, M. H. *J. geol. Soc. Lond.* **136**, 755–770 (1979).
- Hepworth, B. C., Oliver, G. J. H. & McMurtry, M. J. *J. geol. Soc. Lond. Spec. Publ.* **10**, 521–533 (1982).
- Bergström, S. M. *Geol. Förel. Stockh. Förh.* **102**, 377–392 (1980).
- Epstein, A. G., Epstein, J. B. & Harris, L. D. *U.S. geol. Surv. Prof. Pap.* **995**, 1–27 (1977).
- Schiffman, P. & Liou, J. G. *J. Petrology* **21**, 441–474 (1980).
- Bamford, D., Nunn, K., Prodehl, C. & Jacobs, B. *J. geol. Soc. Lond.* **133**, 481–488 (1977).
- Anderton, R., Bridges, P. H., Leeder, M. R. & Sellwood, B. W. *A Dynamic Stratigraphy of the British Isles* (Allen & Unwin, London, 1979).
- Davey, F. J. & Broadbent, M. N. *Z. J. Geol. Geophys.* **23**, 395–406 (1980).
- Turner, F. J. *Metamorphic Petrology—Mineralogical and Field Aspects* 1st Edn (McGraw Hill, New York, 1968).
- Birch, F. *J. geophys. Res.* **65**, 1083–1102 (1960).
- Garrick, R. A. & Hatherton, T. *N.Z. J. Geol. Geophys.* **16**, 973–995 (1973).
- Christensen, N. I. *J. geophys. Res.* **70**, 6147–6164 (1965).
- Walton, E. K. *The British Caledonides* (eds Johnson, M. R. W. & Stewart, F. H.) (Oliver and Boyd, Edinburgh, 1963).

HALL *ET AL.* REPLY—Oliver and McKerrrow accept our conclusions that there are crystalline rocks at shallow depth in the Southern Uplands. Their suggestion that the rocks are greenschist facies metagreywackes, part of an accretionary wedge occupying nearly all the present crustal pile, represents an unlikely possibility rather than a probability more central to all the evidence.

Shots on LISPB¹ and the Otago line² are too far apart to provide good control on shallow velocities. Inspection of the original LISPB data from shot 1 suggests that the velocity of Dalradian greenschist metasediments is significantly less than 6 km s^{-1} at 1 km depth (in the fast direction—along the horizontal banding of the 'flat belt'). From this and other field measurements across banding, we estimate that at 1 km depth these rocks would have a mean velocity of $\sim 5.6\text{ km s}^{-1}$ and anisotropy of less than 7%. The Otago data may be modelled similarly.

Laboratory data³⁻⁵ show average values for anisotropy in small cores of schists and associated rocks at pressures corresponding to 3 km depth to be only 7–9%, although higher individual values are found. Less anisotropy would be expected in the field. The 15% anisotropy sought by Oliver and McKerrrow is likely to require the rocks to have a 40% content of mica⁴ oriented consistently throughout the region.

Thus, Oliver and McKerrrow's claim that greenschist facies metagreywacke at 1 km depth would have a mean velocity of 6 km s^{-1} and 15% anisotropy is at the limit of possibility.

Turning to the geological evidence, it is unlikely that a 'regional' metamorphic transition from a greywacke with little or no fabric to a wholly reconstituted mica schist occurs over only 1 km depth, despite the 'dynamic' example near the Alpine Fault⁶. Oliver and McKerrrow themselves imply elsewhere^{7,8} that the transition in the Southern Uplands takes place over a depth range of 7–12 km.

Oliver and McKerrrow imply that the accretionary wedge occupies more or less the whole crust north of the subduction zone indicated on WINCH⁹ and with at least 10 km already removed by erosion, the stack must have reached a thickness of 40 km or so, far in excess of present-day examples¹⁰.

Xenoliths from post-Caledonide vents in the Southern Uplands are sufficiently similar to those of the Midland Valley to suggest derivation from a similar crust¹¹. It has yet to be established if the English continental crust could supply xenoliths of this type, and whether such crust extends far enough north (interpretation of the Iapetus Suture on WINCH suggests that it does not).

Northward translation¹² of the Southern Uplands is required to account for the missing Ordovician fore-arc basin, and to cover the source of the Silurian conglomerates. We cannot seriously entertain the idea that the substantial thickness of Silurian rocks in the southern Midland Valley came from Cockburnland which, in addition, has to supply sediment to a coeval trench to the south yet is rarely more than 5 km wide¹², and occasionally substantially less¹³. The conglomerates

are unlikely to have come from the top of an accretionary pile because the clasts include acid igneous rock fragments (probably first cycle) and foliated greywacke.

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1. Bamford, D. *et al.* *Geophys. J. R. astr. Soc.* **54**, 43–60 (1978).
2. Davey, F. J. & Broadbent, M. *N.Z. J. Geol. Geophys.* **23**, 395–406 (1980).
3. Birch, F. *J. geophys. Res.* **66**, 2199–2224 (1961).
4. Christensen, N. I. *J. geophys. Res.* **70**, 6147–6164 (1965).
5. Anderson, O. & Liebermann, R. C. in *Physical Acoustics* Vol. 4B (ed. Mason, W. P.) 329–472 (Academic, New York, 1968).
6. Turner, F. J. *Metamorphic Petrology—Mineralogical and Field Aspects* (McGraw-Hill, New York, 1981).
7. Leggett, J. K., McKerrow, W. S. & Eales, M. H. *J. geol. Soc. Lond.* **136**, 755–770 (1979).
8. Oliver, G. J. H. & Leggett, J. K. *Trans. R. Soc. Edinb.* **71**, 235–246 (1980).
9. Brewer, J. A. *et al.* *Nature* **305**, 206–210 (1983).
10. Leggett, J. K. (ed.) *Geol. Soc. Spec. Publ.* **10**, (1982).
11. Upton, B. J. G., Aspen, P. & Chapman, N. A. *J. geol. Soc. Lond.* **140**, 105–121 (1983).
12. Walton, E. K. in *Geology of Scotland* (ed. Craig, G.) 201–227 (Oliver & Boyd, Edinburgh, 1965).
13. Bluck, B. J. *Trans. R. Soc. Edinb. Earth Sci.* **74**, 119–136 (1983).

Geomagnetic reversals

RECENTLY Valet *et al.*¹ presented palaeomagnetic data associated with a pair of reverse-to-normal (R–N) polarity transitions from western Crete. The two transition records, differing in age by about 1.2 Myr, were found to possess very similar directional field behaviour in that the corresponding paths of the virtual geomagnetic pole (VGP) were shown to be nearly identical. The authors then noted that an earlier reported N–R VGP path from Crete², associated with the transition which closely follows in time the younger of the two R–N reversals, is largely discrepant to the other paths. Given these comparisons, the authors conclude, first, that requirements of the standing field model for transition fields³ are not satisfied by these data, and second, that the reversal mechanism in the core may be associated with a definite field configuration over some period of time such that successive reversals of the same sense recorded at the same site will be associated with identical VGP paths.

My purpose here is to explore the above argument further. For the authors' assertion to be strictly valid reversals of opposite sense which occur during a time of assumed invariant transitional field

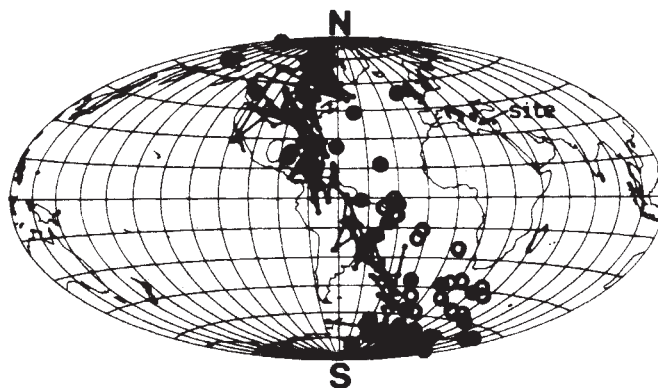


Fig. 1 Paths of the virtual geomagnetic pole associated with three polarity transitions recorded on Crete^{1,2}. The drawn path depicts north poles associated with KS06 R–N transition. The solid circles are north poles associated with the older KS02 R–N transition. The open circles are south poles associated with the younger N–R transition (after refs 1, 2).

characteristics should be observed to possess antipodal VGP paths when recorded at the same site. If, on the other hand, the reversal process is asymmetric such that each transition sense is associated with a different field configuration, then antipodal behaviour probably will not be observed. The question now is: how does the VGP path associated with the N–R transition record² compare with the two R–N transition paths¹?

Figure 1 shows the highly detailed VGP path associated with the younger (KS06) R–N transition¹ along with individual VGPs associated with the older (KS02) R–N transition¹. Also plotted are the individual south VGPs corresponding to the N–R transition². As can be seen in the figure, all three records contain intermediate VGPs which reside along a relatively narrow path west of the site. Indeed, the longitudinal span of this combined path narrows to less than 45° for near-equatorial poles. Furthermore, an east-to-west migration of intermediate VGPs is a common feature of all three paths, where data exist.

For the R–N north pole transition paths to be found in close proximity to the N–R south pole transition path implies that the north VGP paths of the former transitions are roughly antipodal to the north VGP path of the latter transition. This finding suggests that the harmonic content during all three reversals was quite similar apart from a complete change in sign of the transition field associated with the N–R event. Such a situation is consistent with a reversal process whose time-dependent configurational characteristics are independent of transition sense.

In this regard, the generalized flooding model⁴ can successfully accommodate these data through a simulated reversal process which begins and extends from the same localized region in the core for all three polarity transitions from Crete. It is interesting, however, that a pair of recently reported sequential reversals (R–N and N–R) from Kauai, Hawaii⁵, have nearly identical north pole transition

paths. Such a case cannot be strictly explained by the above arguments, but suggests that major changes in the characteristics of the reversal process in the core can occur abruptly between successive geomagnetic transitions.

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1. Valet, J.-P., Laj, C. & Langeris, C. G. *Nature* **304**, 330–332 (1983).
2. Valet, J.-P. & Laj, C. *Earth planet. Sci. Lett.* **54**, 52–63 (1981).
3. Hillhouse, J. & Cox, A. *Earth planet. Sci. Lett.* **29**, 51–64 (1976).
4. Hoffman, K. A. *Earth planet. Sci. Lett.* **44**, 7–17 (1979).
5. Bogue, S. W. & Coc, R. S. *Nature* **295**, 399–401 (1982).

VALET AND LAJ REPLY—We have limited our comparison to two reverse-to-normal (R–N) paths from transitions recorded in the same section. Hoffman remarks that a more complete picture can be obtained using antipodal virtual geomagnetic pole (VGP) paths of N–R transitions. Using the south VGPs relative to a N–R transition recorded in a nearby section at Potamida (which we now call KP02), he concludes that the paths of all three transitions are very similar.

The data for this N–R transition, however, although detailed, are not rigorously classifiable as category 'A' because of lack of points in the second half of the transition¹, as remarked by Hoffman himself². A new detailed sampling with very precise stratigraphic control yields a much more detailed and still unpublished VGP path quite similar to the previous published one, although some differences exist in the equatorial VGPs. A comparison between the KS06 transition VGPs and the south VGPs of this new record is shown in Fig. 2. We believe that this figure shows that the R–N paths from Skouloudhiana and the South Pole N–R path from Potamida are not identical (the difference between the two is smaller

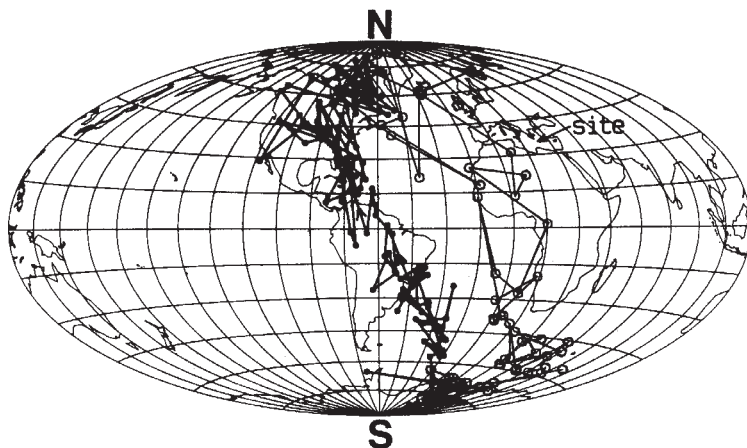


Fig. 2 Paths of virtual geomagnetic poles associated with the KS06 (solid symbols) and KP02 (open symbols) transitions. For KS06 the path depicts north-VGP, for KP02 south VGPs. The two paths appear to be clearly distinct, although an east-to-west migration of intermediate VGPs exists in both of them.

but in our opinion still significant when the old N-R record is used). Moreover, the following remarks can be made.

As pointed out by Hoffman, an east-to-west migration of intermediate VGPs is a common feature of all three paths. In our paper³, we had erroneously thought that this characteristic was related to properties of the Skouloudhiana section. We now agree with Hoffman that it is related to the reversal's mechanism.

The N-R transition in Potamida lies outside the time interval between the two R-N reversals in Skouloudhiana, so the difference observed in the VGP paths indicates that some differences exist between the transitional field configurations relative to KS02 and KS06 on one side and KP02 on the other. It does not, however, yield any information about the N-R transitions occurring during the time interval between KS02 and KS06. Data about these transitions are thus needed to determine whether the differences observed in

the VGPs paths are due to some changes in the reversal mechanism occurring between KS06 and KP02 or whether transitions of opposite sense are always associated with different field configurations.

In Skouloudhiana, unfortunately, the NRM intensity of the sediments at the corresponding stratigraphic horizons is very low, so that measurements are difficult. However, some measurements are now in progress using a cryogenic magnetometer, and we hope to solve this problem.

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1. Valet, J.-P. & Laj, C. *Epsl* **29**, 51-64 (1981).
2. Hoffman, K. *Phil. Trans. R. Soc. A* **306**, 147-159 (1982).
3. Valet, J.-P., Laj, C. & Langereis, C. G. *Nature* **304**, 330-332 (1983).

Factors determining desert dune type

In their study of aeolian dunes, Wasson and Hyde¹ concluded that sand supply is an important determinant of dune type. Their study was based on the assumption that equivalent sand thickness (EST, defined as the ratio of sand volume within dunes to the area of a dune field) can be used to quantify sand supply and sand availability. Our purpose here is to argue that EST is a measure of dune size and shape, not a measure of sand supply. As a result, the technique of equating EST and sand supply can be shown to lead to incorrect conclusions when tested with subaqueous bedforms.

Sand supply and sand availability are general terms that might be loosely used for at least five quantities: (1) the volume of sand within dunes in a unit area of dune field (equal to EST), (2) the total volume

of potentially erodable sand in a unit area of dune field, including sand below the surface that passes through dune troughs, (3) the fraction of a dune field covered by erodable sand, (4) the rate at which sand is supplied to a unit width of dune field from upwind sources, and (5) the rate at which sand accumulates in a dune field (that is, the rate of deposition or erosion, which is equal to the difference between the rates at which sand enters and leaves a unit area of dune field²). The first two quantities have units of length, the third is dimensionless, the fourth has units of area/time, and the fifth has units of length/time. In precise usage, the parameters that measure sand abundance or availability [quantities (1)-(3) above] must be distinguished from the parameters that measure rates of sand transport [quantity (4)] or accumulation [quantity (5)].

Wasson and Hyde state that 'Sand supply and EST are often identical but the

possibility of sand moving through dunes into areas outside dune fields ... means that EST and sand supply may not be exactly the same for highly mobile dune fields, in areas of high wind energy.' Although no single parameter can quantify all five types of sand 'supply' listed above, the statement quoted seems to imply that a quantity measured in units of length (EST) might somehow describe a sediment-transport-rate quantity if the rate is not too high.

Another problem with using EST to measure sand availability is that EST does not include erodable sand that may occur below the surface that passes through dune troughs—a quantity that may represent the bulk of sand in a dune field. In other words, EST is a measure of available sand only if all available sand in a dune field is incorporated within dunes. This assumption, which forms the basis for Wasson and Hyde's analysis, is unsupported, and it is clearly incorrect in areas where dunes deposit cross-stratified beds, because in such areas of deposition, sand is transferred from dunes to underlying beds². The problem of quantifying sand availability might be eliminated by measuring quantity (3) listed above (the fraction of a dune field covered by erodable sand), rather than measuring EST.

Because EST is independent of fluid medium, it is instructive to test Wasson and Hyde's analytical technique with subaqueous bedforms, about which more is known. From a plot of subaqueous bedforms as a function of EST and current variability (like Wasson and Hyde's Fig. 4) one might incorrectly conclude that sand waves occur where sand is abundant, that upper-regime flat beds occur where sand is scarce, and that ripples occur where sand abundance is intermediate. (In reality, flow strength is a more important determinant of what type of subaqueous bedform is produced.) The point of considering subaqueous bedforms is not to argue that aeolian bedforms behave similarly, but rather to demonstrate that, regardless of fluid medium, a correlation between bedform type and EST does not prove that EST (or sand supply) is a determinant of bedform type. Instead, EST may merely reflect bedform type, with bedform type determined by other parameters.

As a result of their EST-based analysis, Wasson and Hyde conclude that: (1) bar-chans occur where there is very little sand, (2) transverse dunes occur where sand is abundant, (3) longitudinal dunes occur where there is little sand, and (4) star dunes occur where sand is abundant. Conclusion (1) is undoubtedly correct, but the other three conclusions are suspect. For example, transverse dunes form in some areas with so little sand cover that underlying rocks or sediments are exposed in deeper parts of dune troughs, and the dune height in some such areas is so small that the mean thickness of erodable sand in the dune field is no more than a few metres.

The conclusion that longitudinal dunes occur where there is little sand is unjustified because the EST measurements on which that conclusion is based are incapable of identifying areas with little available sand (because additional sand may underlie dune troughs).

This question about whether longitudinal dunes can occur where sand is abundant has important ramifications to geologists studying aeolian deposits. If longitudinal dunes can occur only where sand is scarce, they should be unable to produce deposits without causing their own destruction or evolution into other kinds of dunes.

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1. Wasson, R. J. & Hyde, R. *Nature* **304**, 337–339 (1983).
2. Rubin, D. M. & Hunter, R. E. *Sedimentology* **29**, 121–138 (1982).

WASSON AND HYDE REPLY—The aeolian sand both in dunes and swales is that proportion of the total arenaceous sediment, lying either beneath or upwind of a dune field, that has been made available for deflation and dune construction. It follows that equivalent sand thickness (EST) in a dune field in which all of the aeolian sand lies within the dunes is a good measure of the sand made available for dune construction. In dune fields where a large volume of aeolian sand lies in swales, EST is a minimum estimate of the total volume of aeolian sand in the dune field. We do not claim¹, as Rubin believes we do, that EST equals the total arenaceous sediment body that might be deflated in either conditions different from those that have prevailed during the history of the dune field or at some future time. Observations in a number of dune fields clearly show that not all sandy sediments have been deflated to form dunes. In the Thar, Simpson and Kalahari, for example, large stores of non-aeolian sand lie both beneath and upwind of the dunes^{2,3,4}.

Examination of pits, wells and bore-logs both by us and by others shows that significant quantities of aeolian sand lie in swales in fields of star and transverse dunes, very little in fields of longitudinal dunes and none in areas of barchans⁵. The inclusion in estimates of EST of this fraction of aeolian sand would therefore enhance the differences between star/transverse and longitudinal/barchan dunes on our Fig. 4.

We concluded that drift potential (DP, a measure of wind strength) does not correlate with dune type but we now go on to suggest that vegetation and sand moisture have always been modulators of sand transport rates in presently arid to semi-arid dunefields (for example, Australia, Thar, Kalahari). In presently hyper-arid

dune fields (Namib, Sahara, Rub'al Khali) sand transport has always responded directly to the fluid medium. We also note the possibility that EST has been controlled by a number of meteorological and biological factors that differ both between dune fields and through time. The control of subaqueous bedforms by the fluid medium does not have a simple parallel in subaerial dune fields. For example, there is no equivalent of vegetation in river bedforms, and the density differences between solids and the transporting media in the two cases cautions against a simple comparison of the kind suggested by Rubin. Therefore, a test of our ideas about desert dune fields using subaqueous bedforms is casuistry. Rubin notes that he does not wish to infer that aeolian and subaqueous bedforms behave similarly, making his comparison even more puzzling.

One of Rubin's objections to our conclusions is his observation that transverse dunes form in some areas where swales are not covered by aeolian sand, thereby implying low EST. In our Fig. 4 only 1σ is plotted about the mean of EST for each dune type so, clearly, low values of EST were included in our sample. We do not claim that all values of EST in transverse dune fields are higher than all values of EST in longitudinal dune fields. We have used statistical significance as our criterion of difference. Rubin's citing of a few undocumented and unquantified examples unfortunately follows in a long tradition in aeolian geomorphology. We specifically set out to quantify relationships using all of the best data available to us, so as to avoid the selective use of examples.

It is relatively easy to see how the variable RDP/DP might influence dune type. The role of EST is less clear, and so our use of this variable is best viewed as an aid to discrimination between dune types. If transport rates have always been higher in presently hyper-arid dune fields, by comparison with presently more humid dune fields, then it is possible that EST reflects, among other things, the presence or absence of long-distance sediment transport by wind. Star dunes have the highest values of EST and occur in areas in the Sahara where sand flow converges^{5–8}. Simple longitudinal dunes in the Simpson–Strzelecki dune field of Australia have low values of EST and have been derived from sediment sources close by². It is conceivable that EST reflects dune type rather than vice versa. Rubin's comments have highlighted this unresolved problem but we cannot agree with his conclusions.

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1. Wasson, R. J. & Hyde, R. *Nature* **304**, 337–339 (1983).
2. Wasson, R. J. *Z. Geomorph. N. F. Suppl. Bd.* **45**, 85–115 (1983).
3. Heine, K. *Palaeoecol. Afr.* **15**, 53–76 (1982).
4. Wasson, R. J. *et al. Z. Geomorph. Suppl. Bd.* **45**, 117–151 (1983).
5. McKee, E. D. *U.S. geol. Surv. Prof. Pap.* 1052 (1979).
6. Wilson, I. G. *Geogr. J.* **137**, 180–199 (1971).
7. Fryberger, S. G. & Ahlbrandt, T. S. *Z. Geomorph. N. F.* **23**, 440–460 (1979).
8. Mainguet, M. & Callot, Y. *L'Erg de Fachi-Bilma* (CNRS, Paris, 1978).

J. A. MABBUTT COMMENTS—The exchange of views between Wasson and Hyde¹ and Rubin has been useful in clarifying controls over sand availability for dune formation, but in my view does not go far enough in considering how this in turn acts to determine major dune type. This determinant would appear to be the extent of non-dune surface suited to the rapid transmission of saltating sand, relative to the area of sand-trapping dune surface. Where the former is so dominant as to leave the dune accumulations isolated, barchans characteristically develop; where the relationship is such as to allow the continued exposure of downwind-oriented non-dune strips, longitudinal dunes are typical; where, on the other hand, the non-dune floor remains completely buried or revealed only in minor and non-connecting troughs, transverse dunes are found. In many dune fields these relationships occur in sequence downwind towards a basin depression of sand accumulation, and there is a corresponding downwind sequence of dune form. A whole range of circumstances can intervene to determine these relationships locally, including thickness of prior sands available for wind-working into dunes, current aeolian sand supply, the transmissivity of the underlying floor, wind energy and vegetation cover.

Patterns of longitudinal dunes, in which dune and non-dune surfaces are more evenly matched in extent than in the other dune types, are strongly influenced by variations in the nature of this non-dune surface. A recent account of longitudinal dunes in the north-west Simpson Desert² shows that the non-dune 'floor' can vary locally between soft unconsolidated sand (partly wind-worked sandy alluvium?), firm surfaces of coarse-textured alluvium, and hard gibber pavements. The dunes that cross these surfaces may accordingly vary between wind-sculpted forms, sand mounds assembled *in situ*, and transgressive dunes. There is strong evidence that differences in sand-transmissivity between the non-dune 'floors' have contributed to differences in spacing and other pattern attributes.

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1. Wasson, R. J. & Hyde, R. *Nature* **304**, 337–339 (1983).
2. Mabbutt, J. A. & Wooding, R. A. *Z. Geomorph. N. F. Suppl. Bd.* **45**, 51–69 (1983).

Singular mathematics

Brian Goodwin

Catastrophe Theory. By V.I. Arnold. Translated by R.K. Thomas.

Springer-Verlag: 1984. Pp. 78. Pbk DM 16.80, \$8.50.

Mathematical Models of Morphogenesis. By René Thom.

Ellis Horwood/Halsted: 1983. Pp. 305. £25, \$59.95.

"THE nice results of singularity theory are happily not dependent upon the dark mystics of catastrophe theory". Thus V. I. Arnold, a distinguished Russian mathematician, sums up a widely-held professional view of the most extraordinary mathematical development of the century. However, in René Thom's new book, a collection of essays written between 1967 and 1981, Thom goes a long way towards clarifying some of the misunderstandings that lie behind Arnold's judgement, which reflects certain deeply-held prejudices of the scientific community. To put this far-reaching conflict of views into perspective, the remarkable history of catastrophe theory needs to be considered.

Ten years ago, an area of deep and difficult mathematics, involving profound theorems that had aroused the interest of the world's finest mathematicians, suddenly became the topic of newspaper and magazine articles, common-room discussion and party gossip. This resulted from two events: the publication in 1974 of René Thom's first book, *Structural Stability and Morphogenesis*; and E. C. Zeeman's communication to the International Congress of Mathematics in Vancouver in the same year, describing the application of Thom's ideas to a great variety of problems, not only in mechanics and physics but also in biology and the human sciences.

Thom had extended and developed a theory of the mathematician H. Whitney, published in 1955. Whereas classical (i.e. Newtonian) differential calculus considers only smooth, continuous processes, Whitney's theory allowed for a systematic study of the discontinuities, breaks or jumps that can occur in a dynamical process, described by its singularities. Smooth mappings and their singularities are extremely widespread and familiar: flows and wave formation in liquids, meteorological fronts and turbulence, regions of intense illumination due to light (rainbows, curves of bright light at the bottom of swimming pools), the condensation of water vapour, the formation and propagation of defects in crystals, and so on. Following Whitney's basic study, singularity theory developed very rapidly and acted as a focal point for the combination of some of the most abstract areas of mathematics (differential and algebraic geometry and topology, group theory, commutative algebra etc.) with some of the most applied (stability theory, bifurcation theory, geometrical optics etc.)

Thom combined the abstract and the concrete to formulate a comprehensive taxonomy of the singularities that could occur in spaces of four dimensions — three of space and one of time; the world as we know it — suggesting that these singularities are the origins of all natural forms that arise in a sufficiently structured (e.g. polarized) system. Christopher Zeeman located this theory within the broader context of general systems, greatly extending the potential applications of the theory and including the human and social sciences; and he it was who suggested the term "catastrophe theory". The dual offer of a systematic theory describing the genesis of form from Thom, together with Zeeman's examples illustrating its non-linear dynamical applications, suddenly opened a door to an extraordinary world of mathematical modelling. Large numbers of people passed through, but soon many were shouting that they had been offered false goods; that the emperor had no clothes. What did they find in that promised land?

Arnold's clear and often entertaining little book (for which the translator should take some credit) casts a caustically illuminating eye over what he sees as a typically Western marketing phenomenon, summing it up in a quotation from Dorochevich: "I think, my dearest, that all this decadence is nothing more than simply a way to approach tradesmen". His own scientific judgement is severe: "In the majority of serious applications, the singularity is a Whitney cusp [describing basic reversible discontinuities or phase transitions, such as gas condensation and vaporization] and the result was known before the advent of catastrophe theory". This is certainly an over-reaction, for there are thoroughly rigorous applications of the higher-order catastrophes to physical phenomena that depend upon Thom's extension of the Whitney theory, such as the use of the elliptic umbilic to study and predict hydrodynamic stagnation points in the six-roll mill experiments of Berry and Macklin. And, in biology, Zeeman has applied catastrophe theory in original and useful ways to morphogenetic processes in embryos, making predictions and stimulating experiments the results of which are consistent with the model. However, Thom distances himself from such applications, insisting that there is a much more profound use of catastrophe theory than the rigorous quantitative analysis that Arnold takes to be the measure of mathematical

modelling and good science. What is this vision?

The subtitle of Thom's first book was *An Outline of a General Theory of Models*. Despite its title, this second book is, like the first, more about a general theory of modelling than about actual models. Readers may or may not like this style of theorizing, but once accepted the book can be read for what it is: an inspired and imaginative exposition of both a scientific methodology and a research programme, illustrated in diverse contexts (physics, biology, psychology) by using catastrophe theory to sharpen questions rather than to obtain answers to specific problems. Like a piece of classical theatre, the climax of the book comes in the middle, Chapters 7 and 8, containing Thom's defence of his conception of his theory. With a gesture characteristic of the central image of catastrophe theory, the bifurcation set of the cusp, Thom opens our imaginations with a perceptive discussion of the aims of science and the nature of space-time, magic and geometry, and then brings these perennial themes into sharp focus on a problem for which he offers, not an answer, but a programme of investigation.

The discussion is centred on the struggle to unify action with understanding, the former aiming to resolve local problems while the latter seeks the universal or the global. Thom argues that the great scientific successes (Newtonian gravitation, electromagnetism, quantum mechanics, relativity theory) are non-local theories that describe the magic of action-at-a-distance in terms of strictly controlled local manifestations: for example, Maxwell's equations localize electromagnetism; Einstein rendered gravitation local. Theories that have no "magical" non-local elements, argues Thom, do not introduce new possibilities of action. "Catastrophe theory, anti-magic *par excellence*, pushes the principle of locality to the extreme: this is without doubt why it hardly offers new practical possibilities". Must it not, then, also fail to provide insight or understanding, since it has no global properties? Here Thom offers a research programme the objective of which is to construct the global from the local by a procedure in which the *Urbild* of spatiality, the generative principle of space, is an organizing centre that unfolds, like the big bang model in cosmology or the embryo which develops from the egg. This is clearly congenial to catastrophe theory which is precisely about the unfolding of form from organizing germs in space-time, about those singularities which contain in intensely localized form the potential for generating a specific, extended structure. But a rigorous mathematical theory of such a generative procedure is as yet non-existent.

The absence of an appropriate theory, however, does not stop Thom from exploring implications for research in biology,

linguistics, semiotics and semantics. His cognitive programme is to geometrize thought itself, using the elementary catastrophes as logoi, the algebraico-geometrical structures which stabilize concepts in mental activity space. Semantics is based upon approximate isomorphisms between logoi of material beings and those of the corresponding concepts, and so mind perceives real properties of objects which are the "bearers of meaning". His objective is then "to create a theory of meaning whose nature is such that the act itself of knowing is a consequence of the theory; i.e., when an appearance appears to us as a bearer of meaning, we will know why and to what formal facts to attribute it".

What people have primarily reacted against in Thom's writing is the highly discursive style combined with the sharply-defined analytical tools of abstract mathematics. We are used to having mathematics applied to specific problems, leading to solutions. Thom applies it to perennial philosophical and scientific problems, leading to further questions. Clearly catastrophe theory works in physics, where singularity theory worked before, and it is clear how to apply it following that lead. This is the path of wisdom Arnold recommends and illustrates. No one has yet shown how to apply catastrophe theory to the general problems of morphogenesis, cognition and the new physics in the style of a generative science of form suggested by Thom. Until it is tried, it seems premature to condemn it — but no one should be in any doubt about the difficulty of such a programme. □

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Thin-necked upright display posture of the male black-billed capercaillie. The drawing is reproduced from Paul A. Johnsgard's *The Grouse of the World*, published by the University of Nebraska Press in the United States and Croom Helm in Britain. Price is \$42.50, £30.

Sense of energy

Ian Smart

Sun Traps. By John Elkington.
Penguin/Viking: 1984. Pp.400.
Pbk £3.95, \$6.95.

EMBEDDED in Mr Elkington's chapters, on fossil fuels and nuclear power as well as renewable energy sources, there is a feast of fact, from a photovoltaic hat to the mass of firewood needed to dry a kilogram of ginger. Like many "popular" energy books, this one tries to handle an astonishing range of topics by assembling anecdotes about all of them, larded with numbers and speckled with names. It is described as a "comprehensive survey on solar power and future energy". But honest efforts to follow the thread of argument through the labyrinth are repeatedly foiled: diverted into yet another garden of colourful trivialities, or distracted by dramatic writing in the "little-did-he-know-that" tradition.

None of this prevents Mr Elkington from being informative. Nor, which is more important, does it hide his good sense — although it sometimes overshadows it. He begins by pointing out that technical feasibility is no substitute for — or index to — economic competitiveness. And he ends with a studiously moderate estimate of the contribution renewable sources will make to world energy supply in any of our lifetimes. His assessment could only have been reinforced by more persistent attention to

economics. As it is, its moderation serves to highlight the euphoric exaggeration by so many of the sun, wind and wave enthusiasts from whom he quotes: a habit of hyperbole matching the former excesses of nuclear apostles and inviting a similar fate.

Mr Elkington himself gets the balance about right, by characterizing as only slightly optimistic a Worldwatch Institute projection that the renewable contribution to global energy supply will rise from 16.5% in 1980 to 23.9% in 2000 — with three-quarters of the increase coming from hydroelectricity, geothermal power and fuelwood. He is no less right to insist that solar-based energy, exploited at reasonable cost and applied in appropriate ways, must make a larger contribution to human welfare. Unfortunately, he barely touches on the fundamental problems involved: the political and economic tension between centralized renewable energy systems, using "high" technology, and decentralized networks, seeking social as well as technical simplicity; the need to choose between ecologically incompatible interests (as between nature conservation and biomass development); and the ultimate dilemma of whether energy should be converted to accommodate society's demands, or social preferences adapted to efficient energy conversion. It is a pity he did not sacrifice more of his anecdotal scrapbook to exploring them. For, through these thickets of lore, there shine rays of a judicious realism. □

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Walk in the gardens

George Oster

An Introduction to the Physical Chemistry of Biological Organization.
By A.R. Peacocke.
Clarendon: 1983. Pp.302. £35, \$65.

FORMALISMS are seductive sirens, for they seem to unify so many disparate threads and they promise deep insights using only mathematical manipulations. Of these seductresses, surely thermodynamics is the most alluring. One of its foremost practitioners, the late Aharon Katchalsky, once observed wryly that "it is such a marvelous tool: you don't know what you put in, and you don't understand what you get out. But you know it must be right!".

In physics and chemistry, thermodynamics has performed as advertised; however, in biology it has yet to live up to its billing. By now, no one expects living systems to disobey the laws of thermodynamics, but these tenets are crude and vague constraints when applied to such systems, and they provide very little insight into the operation of biological entities. Nonequilibrium thermodynamics, by

extending the venue of classical thermostatics into the realm of dynamics, promised to deliver new understanding and unifying concepts which could shed light on the mysteries of biological organization. But has it? Well, that is a matter of dispute, and reasonable scientists can disagree — although on this subject they usually do so with righteous zeal.

Clearly, Professor Peacocke feels that the sirens' song is worth pursuing, for he devotes the first half of his book to the subject of "Thermodynamic Interpretations of Living Systems". This label is a bit misleading, for discussion of living systems is rarely opened. Only in a few pages of these chapters is any application to specific biological problems attempted, and these attempts consist largely of recasting several biochemical models in the language of network thermodynamics. Beyond a certain notational and conceptual simplification — not to be sneezed at, to be sure — it is not clear that any new understanding emerges.

Chapter 1 commences with a discursive and philosophical excursion into the muddy waters of "complexity" and "hierarchies", finally enunciating the "central problem of biology" as (according to H. Pattee): "... the origin and operation of

the hierarchical constraints which harness matter to perform coherent functions". Well, it is true that we would like to know how things work, but recasting the problem in social science lingo doesn't enlighten me. Indeed, Professor Peacocke has collected together a virtual thesaurus of quotations which he employs to illustrate and entertain as he leads us in Chapters 2 and 3 through the various formalisms of irreversible thermodynamics developed by the disciples of Prigogine and of Katchalsky. And an unusually clear tour it is, although somewhat sparse in mathematical detail.

Part II of the book is entitled "Kinetic Interpretations of Living Systems". Here there is more contact with specific situations, and less development of formalisms — perhaps because the subject matter is more inherently mathematical. However, the applications are more chemical than biological, and the author's treatment barely ripples the surface of some quite deep mathematical waters. For example, in Chapter 4, "The Kinetics of Biological Organization", he describes various chemical schemes which, when combined with diffusion, can produce spatial concentration patterns. He mentions the best work in this field, notably that of Meinhardt and Murray, but the treatment is mostly descriptive, albeit lucid. It seems to me that Professor Peacocke frequently opted for a philosophical digression rather than a detailed description of how to perform the calculations required to test whether a model can exhibit spatial instabilities. These recipes are admittedly complicated, but their absence points out the major problem with the book: it is too sketchy on detail to serve as a textbook, but too mathematical to provide biologists with an introduction to a number of active research areas in physical chemistry. However, I found the book easy and pleasant reading and the wealth of references to be most useful. Chapter 5, which describes theories of macromolecular evolution, especially that of M. Eigen, is especially clear. The concluding chapter, "The Interpretation of Biological Complexity", is, like the introduction, a collection of philosophical quotations from various authors on how to best tackle the problems of biological organization.

I am finally left with the feeling that I was glad to have read Professor Peacocke's book; but I am not sure what the wider audience for it will be. Biologists will not find that the mathematics leads them to new experiments, and physical chemists will have to consult more detailed treatments to acquire the techniques; but both may find some agreeable perspectives from which to view their work. Perhaps the book should be read for what it is: a philosophical walk through the gardens that Professor Peacocke finds most beautiful. □

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Poet in the field

Richard Mabey

The Natural History Prose Writing of John Clare.

Edited by Margaret Grainger.

Clarendon: 1984. Pp.397. £35, \$67.

THE STANDING of the agricultural labourer-turned-poet, John Clare (1793–1864), as a nature writer has never been higher. Yet his reputation as a *scientific* naturalist has until now rested on his adding to the Northamptonshire lists 65 first county records for birds and more than 40 for plants. These figures are the more remarkable when you consider that they were worked out retrospectively from his poems, and are backed up by nothing more substantial than the absolute conviction his poetry carries of being based on personal experience. Poets might be inclined towards invention, but not, so Clare mythology insists, one who was also a peasant.

Now, thanks to an impressive and sympathetically edited work of scholarship by Margaret Grainger, we have access to an apparently more objective record of Clare's field observations. Mining the seemingly inexhaustible supply of old notebooks, letters, scribbled envelopes and book jackets in the Clare manuscript collection in Peterborough Museum, Ms Grainger has pieced together the bulk of his natural history prose written between 1820 and 1850. Some is little more than random jottings, but there are also the first notes towards a planned *Natural History of Helpstone* (he had read Gilbert White) which he planned to call *Biographys of Birds & Flowers*.

I said "apparently more objective" above because the first thing that strikes you about this collection is how adept this man, supposedly blessed or imprisoned by an "innocent eye", was at *choosing* how he wrote, and at moving between scientific and vernacular modes of classification and expression. In a detailed letter to the publisher Hessey about Elizabeth Dent's *Flora Domestica*, for instance, he writes:

Cardamine page 74 this is call'd lilac with us as well as 'ladysmock' but I never heard it call'd cuckoo in my life otherwise then [sic] by books — the wood anemone is also call'd 'lady smock' by children what the common people call 'cuckoo' with us is one which is a species of the 'Orchis'. . . .

This upstart and professional tone was not at all what his publishers wanted from their tame ploughboy, and it is no surprise that his proposed *Natural History* never came to fruition.

But his working journal (kept between 1824 and 1828) does survive, and is perhaps the most fascinating part of this book — not so much because of the accuracy and rapt attention of his descriptions, but because, just as in the poems, they are

worked without any change of perspective into accounts of his own feelings. The life of the fields and of Clare's imagination are one and the same. He saw himself, crucially, as *part* of nature. On 10 September 1824, he is feeling unwell, and records a sorrowful note on the swallows congregating in the walnut trees and chattering as "if they were telling their young stories of their long journey to cheer & check fears". The next day he is writing an essay on "the sexual system of plants" for his *Natural History*, and reading a review of Goethe in the *London Magazine*. And in the journal's appendix there is an



John Clare: *part* of nature.

electric account of a meeting with a "will o'whisp":

it blazd out like a whisp of straw & made a crackling noise like straw burning which soon convinced me of its visit the luminous haloo that spread from it was of a mysterious terrific hue & the enlargd size & whiteness of my own hands frit me the rushes appeard to have grown up as large & tall as walebone whips & the bushes seemd to be climbing the sky. . . .

These extracts are classic Clare, troubled, sensitive, highly literate despite the idiosyncratic grammar. Ms Grainger has done for the prose what John Barrell did some years ago for the poetry, demonstrating that its style and perspective are as much a consequence of Clare being jerked "out of his knowledge" by self-education and the disorientating effects of enclosure, as of the depth of his local roots. And in so doing her book raises questions about whether there is any essential difference between "subjective" and "objective" knowledge, and for that matter between poetic and scientific description. Towards the end of the book is Clare's list of the orchids he knew from Helpstone parish (16 species, remarkably). In the precise and affectionate detailing of the places in which each species grew — early marsh orchid was plentiful "on a spot called Parkers Moor near Peasfield-hedge & on Deadmoor near Sheef green . . . but these places are now all under plough" — it is both a haunting evocation of the inner geography of Clare's experience, and the most exact account of locations that any historical botanist could demand. □

Richard Mabey is a writer on countryside matters and landscape history, and is currently working on a biography of Gilbert White.

Ice flow and applied mathematics

Charles F. Raymond

Theoretical Glaciology.

By Kolumban Hutter.

Reidel/MTP Press: 1983. Pp.510.

Dfl. 240, \$104, £60.95.

THE modern phase of glaciology opened in the late 1940s when a small contingent of physicists became interested in the motion of glaciers and ice sheets. Typical of physicists, they emphasized a research method which isolates characteristic parts of glacier behaviour to illuminate essential physical relationships, and they initiated a progressive growth in understanding that continues today. In the past few years a group with a strong applied mathematical bent has come upon the scene. These scientists like to state problems in their fullest mathematical generality, simplifying them only to the extent needed to make a solution feasible. They bring a new analytical expertise to glaciology.

Although the physical and the applied mathematical approaches are essential complements to one another, in the current glaciological community there is something of a communication gap between people who identify with one or the other. Kolumban Hutter is identified firmly with the applied mathematical viewpoint and through this book advocates it. However, Hutter also recognizes the communication gap and tries to bridge it. He has succeeded in writing a unique and rich synthesis that will please any glaciologist who makes the effort to delve into it.

In broad plan the book proceeds from a brief development of general concepts of continuum physics (Part I), to their application to specific problems in the flow of glaciers and ice sheets (Part II). Along the way there is a narrowing of complexity in the physics and a step-by-step broadening of complexity in geometry and time dependence of boundary conditions.

Part I (about one quarter of the book) covers balance laws for mass, momentum, energy and entropy, the continuity (or jump) conditions for these quantities at boundaries, and constraints on the form of constitutive relations. These principles provide the rigorous framework for describing realistic mechanical and thermodynamic behaviour of both polar

(sub-freezing) and temperate (wet) ice.

Part II deals with ice motion in geometries of increasing complexity, starting with a parallel-sided slab and progressing to three-dimensional problems. In addition, the problem of determining the geometry of the free upper surface under steady or time-varying climate is examined. While these topics have been considered in earlier books and review articles, Hutter's development is dramatically different and more expansive. Although a number of mathematical techniques are employed, perturbation expansions are a recurring theme. These analyses impressively demonstrate how far one can proceed with analytical methods, despite non-linearity of ice creep, a basal sliding law, and coupled mass and heat flow.

In the second part in particular, Hutter writes with clarity and confidence. Each chapter is crafted to stand independently yet fit into the whole. The balance of text and equations enables a reader to follow

the thread of the mathematical development without necessarily studying all of the manipulative details.

Theoretical Glaciology does not attempt to provide much information about real glaciers or ice sheets. It also does not cover the physics of glacier sliding or water motion through glaciers, which are topics of great importance and considerable research activity. By itself, then, it is not suitable as a text for a general course in glaciology. However, for a graduate-level theoretical course in glacier dynamics it is excellent, and any glaciologist working in glacier dynamics will find access to the book to be essential. Although it remains to be seen whether *Theoretical Glaciology* will open a new era of glaciological research, it will undoubtedly set a new standard for mathematical analysis of ice flow. □

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Taxonomy of vision

David J. Tolhurst

Parallel Processing in the Visual System:

The Classification of Retinal Ganglion Cells and its Impact on the Neurobiology of Vision.

By Jonathan Stone.

Plenum: 1983. Pp.438. \$55, £42.35.

THE mammalian retina contains several populations of ganglion cells. Each population extracts information about different aspects of the visual world and conveys that information differentially to the brain. The notion of parallel processing of visual information has become so pervasive that anatomists, physiologists and psychophysicists are increasingly tempted to explain any observation in such terms. There is now a large mass of data which needs critical examination, partly to distinguish the real from the spurious and partly to resolve the many discrepancies.

It is disappointing, therefore, that Jonathan Stone has not chosen to provide a straightforward review of that published data. Rather, he uses the literature as a vehicle for expressing his views that the classification of ganglion cells is in itself the primary goal and that the central processing of visual information can be understood only in terms of parallel processing. There is no systematic description of the several populations of ganglion cell, and the references to the literature are often so cryptic that arguments cannot be followed unless the reader has a good knowledge of what has been written on the subject. It is unclear why some observations are discussed at length whilst others are ignored. The book, then, is not an introduction to the subject.

Stone's first objective is to classify gang-

lion cells. He argues with some merit that the taxa should be based on an assessment of the cells' overall behaviour rather than on one 'key' feature of behaviour. However, he does not explain how one should classify a cell when individual aspects of its behaviour seem to conflict. The final climax of the book is the proposal that X and Y cell classes are not at the same taxonomic level as the W cell class. Rather, X and Y cells are subclasses of a single XY class. The questions of why there are two kinds of cell and why their behaviour differs in particular ways is hardly considered.

The notion of parallel processing has certainly had an impact on the neurobiology of vision: there are many papers that mention it. However, Stone does not make clear how visual science would have floundered without the invocation of X, Y and W cells. The central processing of visual information is described very firmly from the contention that there must be parallel processing of the information conveyed by X, Y and W cells. Deviations from the theme are dismissed. For instance, the X/Y/W classification is orthogonal to Hubel and Wiesel's classification of simple and complex cells in the visual cortex. Stone implies that the simple/complex classification must, therefore, be unsatisfactory and concludes: "the question of the organization of the visual cortex to process the activities of different groups of ganglion cells is being successfully pursued without employing the 'simple'/'complex' distinction among cortical cells".

Perhaps Stone's use of quotation marks in describing simple and complex cells reveals the degree of objectivity in his discussion of parallel processing in the visual system. This is a book for the converted. □

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New in paperback

• *The Myths of Human Evolution* by Niles Eldredge and Ian Tattersall. Publisher is Columbia University Press, price is \$10.95 (in the United States and elsewhere). For review see *Nature* 303, 90; 1983.

• *Microman: Living and Growing with Computers* by Gordon Pask and Susan Curran. Published by Century, London, price £4.95. For review see *Nature* 302, 771; 1983.

Biological Sciences

Genes: Structure and Expression. Horizons in Biochemistry and Biophysics, Vol.7. A.M. KROON, E. QUAGLIARELLO and F. PALMIERI (eds). Wiley: 1983. Pp.362. ISBN 0-471-90264-0. £24.

Genetic Resources of Forage Plants. J.G. McIVOR and R.A. BRAY (eds). CSIRO: 1983. Pp.340. ISBN 0-643-03462-5. \$30.

Genetics, Evolution, and Disease. D.H. O'ROURKE, G.M. PETERSEN and F.E. JOHNSTON (eds). Alan R. Liss: 1983. Pp.136. ISBN 0-8451-0227-3. £22.

Genetik. By F. KAUEWITZ. Verlag Eugen Ulmer: 1983. Pp.443. Pbk ISBN 3-8001-2451-3. DM 29.80.

Gerade und Ungerade: Die Asymmetrie des Gehirns und der Zeichensysteme. By V.V. IVANOV. S. Hirzel Verlag: 18. Pp.221. Pbk ISBN 3-7776-0375-9. DM 28.

Gorilla Behavior. By T.L. MAPLE and M.P. HOFF. Van Nostrand Reinhold: 1983. Pp.290. ISBN 0-442-25152-1. \$32.

Handbook of Animal Radio-Tracking. By L.D. MECH. University of Minnesota Press: 1983. Pp.107. Hbk ISBN 0-8166-1222-6; pbk ISBN 0-8166-1221-8. Hbk \$25; pbk \$9.95.

Handbook of Experimental Pollination Biology. C.E. JONES and R.J. LITTLE (eds). Van Nostrand Reinhold: 1983. Pp.558. ISBN 0-442-24676-5. \$46.50.

Handbook of Neurochemistry, 2nd Edn, Vol.5: Metabolic Turnover in the Nervous System. A. LAJTHA (ed.). Plenum: 1983. Pp.495. ISBN 0-306-41323-X. \$69.50.

Hormonal Proteins and Peptides, Vol.11: Gonadotropic Hormones. C.H. LI (ed.). Academic: 1983. Pp.188. ISBN 0-12-447211-7. \$42.

Hormonal Regulation of Testicular Descent. Advances in Anatomy, Embryology and Cell Biology, Vol.81. By U.-F. HABENICHT and F. NEUMANN. Springer-Verlag: 1983. Pp.55. Pbk ISBN 3-540-12439-X/0-387-12439-X. DM 48, \$19.10.

Hormone Action, Part F: Protein Kinases. Methods in Enzymology, Vol.99. J.D. CORBIN and J.G. HARDMAN (eds). Academic: 1983. Pp.433. ISBN 0-12-181999-X. \$49.

The Hunting Animal. By F. RUSSELL. Harper & Row: 1983. Pp.211. ISBN 0-06-015106-4. \$13.95.

Hybridomas and Cellular Immortality. B.H. TOM and J.P. ALLISON (eds). Plenum: 1983. Pp.306. ISBN 0-306-41467-8. \$35.

The Hypothalamo-Pituitary Control of the Ovary and the Factors which Interact with It, Vol.3. By J.S.M. HUTCHINSON. Eden Press: 1983. Pp.231. ISBN 0-88831-118-4. £33.25.

Inorganic Plant Nutrition. By H.G. GAUCH. Dowden, Hutchinson & Ross: 1982. Pp.488. ISBN 0-87933-003-1. \$49.50.

Intestinal Transport: Fundamental and Comparative Aspects. M. GILLES-BAILLIEN and R. GILLES (eds). Springer-Verlag: 1983. Pp.375. ISBN 3-540-12430-6/0-387-12430-6. DM 98, \$38.90.

An Introduction to the Physical Chemistry of Biological Organization. By A.R. PEACOCKE. Oxford University Press: 1983. Pp.302. ISBN 0-19-855359-5. £35.

A Key to the Adults of the British Ephemeroptera with Notes on Their Ecology. J.M. ELLIOTT and U.H. HUMPECH. Freshwater Biological Association: 1983. Pp.101. Pbk ISBN 0-900386-45-2. £4.50.

Key Papers on Fish Populations. D.H. CUSHING (ed.). IRL: 1983. Pp.405. Pbk ISBN 0-904147-58-4. £21, \$42.

Languages in Primates: Perspectives and Implications. Springer Series in Language and Communication, Vol.11. J. DE LUCE and H.T. WILDER (eds). Springer-Verlag: 1983. Pp.198. Hbk ISBN 0-387-90798-X/3-540-90798-X; pbk ISBN 0-387-90799-8/3-540-90799-8. Hbk DM 64, \$25.40; pbk np.

Learning in Animals. R.W. HENDERSEN (ed.). Hutchinson Ross: 1983. Pp.350. ISBN 0-87933-348-0. \$46.

Light and Photosynthesis in Aquatic Ecosystems. By J.T.O. KIRK. Cambridge University Press: 1983. Pp.401. ISBN 0-521-24450-1. £37.50, \$74.50.

Liposome Letters. A.D. BANGHAM (ed.). Academic: 1983. Pp.440. ISBN 0-12-077780-0. £17.50, \$29.

Marine Ecology: A Comprehensive, Integrated Treatise on Life in Oceans and Coastal Waters, Vol.5, Part 2. O. KINNE (ed.). Wiley: 1983. Pp.643-1090. ISBN 0-471-90159-8. £45, \$85.

Membranbiochemie. By H. SANDERMANN. Springer-Verlag: 1983. Pp.131. Pbk ISBN 3-540-12594-9/0-387-12594-9. DM 44, \$17.10.

The Merck Index: An Encyclopedia of Chemicals, Drugs and Biologicals, 10th Edn. M. WINDHOLZ (ed.). Merck: 1983. Pp.1,463 + appendix. ISBN 911910-27-1. Np.

Metal Ions in Biological Systems, Vol.16: Methods Involving Metal Ions and Complexes in Clinical Chemistry. H. SIGEL (ed.). Marcel Dekker: 1983. Pp.432. ISBN 0-8247-7038-2. SwFr. 203.

Molecular Basis of Thyroid Hormone Action. J.H. OPPENHEIMER and H.H. SAMUELS (eds). Academic: 1983. Pp.498. ISBN 0-12-527560-9. \$58.

Molecular Biology of Polyomaviruses and Herpesviruses. By B.V. PERBAL, H.K. LINKE and G.C. FAREED. Wiley: 1983. Pp.247. ISBN 0-471-05058-X. £40.60, \$56.95.

The Mollusca, Vol.3: Development. N.H. VERDONK, J.A.M. VAN DEN BIGGELAAR and A.S. TOMPA (eds). Academic: 1983. Pp.352. ISBN 0-12-751403-1. \$39.50.

The Mollusca, Vol.4: Physiology, Part 1. A.S.M. SALEUDDIN and K.M. WILBUR (eds). Academic: 1983. Pp.523. ISBN 0-12-751404-X. \$65.

Muscle and Nonmuscle Motility, Vol.1. A. STRACHER (ed.). Academic: 1983. Pp.372. ISBN 0-12-673001-6. \$45.

Muscle and Nonmuscle Motility, Vol.2. A. STRACHER (ed.). Academic: 1983. Pp.211. ISBN 0-12-673002-4. \$34.50.

Neurobiology of the Hippocampus. W. SEIFERT (ed.). Academic: 1983. Pp.676. ISBN 0-12-634880-4. £40, \$69.50.

Neurobiology of the Trace Elements, Vol.1: Trace Element Neurobiology and Deficiencies. I.E. DREOSTI and R.M. SMITH (eds). Humana: 1983. Pp.374. ISBN 0-89603-046-6. \$49.50 (US), \$59.50 (elsewhere).

Neurobiology of the Trace Elements, Vol.2: Neurotoxicology and Neuropharmacology. I.E. DREOSTI and R.M. SMITH (eds). Humana: 1983. Pp.520. ISBN 0-89603-047-4. \$49.50 (US), \$59.50 (elsewhere).

Normal and Abnormal Epidermal Differentiation. Current Problems in Dermatology, Vol.11. M. SEIJI and I.A. BERNSTEIN (eds). Karger: 1983. Pp.346. Pbk ISBN 3-8055-3752-2. SwFr. 166, DM 199, \$99.50.

Numerical Taxonomy. NATO ASI Series, Vol.1. J. FELSENSTEIN (ed.). Springer-Verlag: 1983. Pp.644. ISBN 3-540-12293-1/0-387-12293-1. DM 149, \$57.90.

Olfactory Imprinting and Homing in Salmon: Investigations into the Mechanism of the Imprinting Process. Zoophysiology, Vol.14. By A.D. HASLER and A.T. SCHOLZ. Springer-Verlag: 1983. Pp.134. ISBN 3-540-12519-1/0-387-12519-1. DM 69, \$26.80.

Applied Biological Sciences

Advances in Applied Biology, Vol.8. T.H. COAKER (ed.). Academic: 1983. Pp.24. ISBN 0-12-040908-9. £29.50, \$49.50.

Advances in Epileptology: The 14th Epilepsy International Symposium. M. PARSONAGE *et al.* (eds). Raven: 1983. Pp.342. ISBN 0-89004-943-2. \$53.50.

Advances in Potato Pest Management. J.H. LASHOMB and R. CASAGRANDE (eds). Hutchinson Ross: 1982. Pp.288. ISBN 0-87933-407-X. \$22.

Advances in Veterinary Science and Comparative Medicine, Vol.27. C.E. CHORNELIUS and C.F. SIMPSON (eds). Academic: 1983. Pp.519. ISBN 0-12-039227-5. \$58.

Anorexia Nervosa: Recent Developments in Research. Neurology and Neurobiology, Vol.3. P.L. DARBY *et al.* (eds). Alan R. Liss: 1983. Pp.472. ISBN 0-8451-2702-0. £73.

Radioprotectors and Anticarcinogens. O.F. NYGAARD and M.G. SIMIC (eds). Academic: 1983. Pp.773. ISBN 0-12-523360-4. \$49.50.

Recent Advances in Bone Marrow Transplantation. R. P. GALE (ed.). Alan R. Liss: 1983. Pp.932. ISBN 0-8451-2606-7. £75.

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The Scientific Principles of Crop Protection, 7th Edn. By H. MARTIN and D. WOODCOCK. Edward Arnold: 1983. Pp.486. ISBN 0-7131-3467-4. £35.

Sex Selection of Children. N.G. BENNETT (ed.). Academic: 1983. Pp.256. ISBN 0-12-088860-2. \$35.

Survey of Drug Research in Immunologic Disease, Vol.3: Noncondensed Aromatic Derivatives, Part II. Karger: 1983. Pp.582. ISBN 3-8055-3687-9. SwFr. 490, DM 588, \$293.50.

Textbook of Contraceptive Practice, 2nd Edn. By M. POTTS and P. DIGGORY. Cambridge University Press: 1983. Pp.467. Hbk ISBN 0-521-24934-1; pbk ISBN 0-521-27085-5. Hbk £30, \$59.50; pbk £12.50, \$24.95.

Thirteenth International Cancer Congress, Parts A-E. Proceedings of the 13th International Meeting held September 1982, Seattle. E.A. MIRAND, W.B. HUTCHINSON and E. MIHICH (eds). Alan R. Liss: 1983. Set ISBN 0-8451-0132-3. Pt A, Current Perspectives in Cancer, pp.228, £26; Pt B, Biology of Cancer Vol.1, pp.462, £47; Pt C, Biology of Cancer Vol.2, pp.504, £50; Pt D, Research and Treatment, pp.542, £55; Pt E, Cancer Management, pp.532, £55.

Toxic Hazards in Food. By D.M. CONNING and A.B.G. LANDSDOWN. Croom Helm: 1983. Pp.297. ISBN 0-7099-0253-0. £22.50.

Toxicology in the Use, Misuse and Abuse of Food, Drugs and Chemicals. Proceedings of the Meeting held March 1982, Tel Aviv. P.L. CHAMBERS, S. GITTER and C.M. CHAMBERS (eds). Springer-Verlag: 1983. Pp.403. Pbk ISBN 3-540-12393-X/0-387-12393-X. DM 127, \$50.50.

Tropical Rain Forest: Ecology and Management. Special Publication no. 2 of the British Ecological Society. S.L. SUTTON, T.C. WHITMORE and A.C. CHADWICK (eds). Blackwell Scientific: 1983. Pp.498. ISBN 0-632-01142-4. £28.50.

Viral Hepatitis: Standardization in Immunoprophylaxis of Infections by Hepatitis Virus. Developments in Biological Standardization, Vol.54. Karger: 1983. Pp.590. Pbk ISBN 3-8055-3826-X. SwFr. 135, DM 162, \$81.

Zinc Deficiency in Human Subjects. A.S. PRASAD *et al.* (eds). Alan R. Liss: 1983. Pp.284. ISBN 0-8451-0129-3. £28.

Psychology

A Clinical Pharmacology in Psychiatry: Bridging the Experimental-Therapeutic Gap. L.F. GRAM *et al.* (eds). Macmillan Press, London: 1983. Pp.413. ISBN 0-333-34724-2. £62.

Cognitive Therapy with Couples and Groups. A. FREEMAN (ed.). Plenum: 1983. Pp.343. ISBN 0-306-41149-0. \$35.

Freud and Modern Psychology, Vol.2: The Emotional Basis of Human Behavior. By H.B. LEWIS. Plenum: 1983. Pp.237. ISBN 0-306-4132-9. \$25.

Hearing — Physiological Bases and Psychophysics. Proceedings of the 6th International Symposium held April 1983, Germany. R. KLINKE and R. HARTMANN (eds). Springer-Verlag: 1983. Pp.388. ISBN 3-540-12618-X/0-387-12618-X. DM 68, \$27.

New Directions in Tardive Dyskinesia Research. Modern Problems of Pharmacopsychiatry, Vol.21. J. BANNET and R.H. BELMAKER (eds). Karger: 1983. Pp.222. ISBN 3-8055-3735-2. SwFr. 126, DM 151, \$75.50.

Psychosomatic Medicine: Theoretical, Clinical and Transcultural Aspects. A.J. KRAKOWSKI and C.P. KIMBALL (eds). Plenum: 1983. Pp.830. ISBN 0-306-41279-9. \$95.

Social and Personality Development: Infancy through Adolescence. By W. DAMON. W.W. Norton: 1983. Pp.392. Hbk ISBN 0-393-01742-7; pbk ISBN 0-393-95248-7. Hbk np; pbk £11.50.

Social Skills Assessment and Training with Children: An Empirically based Handbook. By L. MICHELSON *et al.* Plenum: 1983. Pp.268. ISBN 0-306-41234-9. \$35.

Speech and Situation: A Psychological Conception of Situated Speaking. By T. HERRMANN. Springer-Verlag: 1983. Pp.185. ISBN 3-540-12768-2/0-387-12768-2. DM 64, \$24.90.

Sociology

Between Basti Dwellers and Bureaucrats: Lessons in Squatter Settlement Upgrading in Karachi. J.W. SCHORI, J.J. VAN DER LINDEN and K.S. YAP (eds). Pergamon: 1983. Pp.305. Hbk ISBN 0-08-027971-6; pbk ISBN 0-08-030229-7. Hbk np; pbk £9.50, \$17.

The Expanding Circle: Ethics and Sociobiology. By P. SINGER. Oxford University Press: 1983. Pp.189. Pbk ISBN 0-19-283038-4. £3.95.

Social Science Research and Climate Change: An Interdisciplinary Appraisal. R.S. CHEN, E. BOULDING and S.H. SCHNEIDER (eds). Reidel: 1983. Pp.255. ISBN 90-277-1490-8. Dfl.100, \$43.50.

History of Science

Eddington: The Most Distinguished Astrophysicist of his Time. By S. CHANDRASEKHAR. Cambridge University Press: 1983. Pp.64. ISBN 0-521-25746-8. £7.50, \$12.50.

History and Philosophy of Science: Selected Papers. Annals of the New York Academy of Sciences, Vol.412. J.W. DAUBEN and V.S. SEXTON (eds). New York Academy of Sciences: 1983. Pp.168. Hbk ISBN 0-89766-217-2; pbk ISBN 0-89766-218-0. Hbk np; pbk \$33.

The Philosophical Naturalists: Themes in Early Nineteenth-Century British Biology. By P.F. REHBOCK. University of Wisconsin Press: 1983. Pp.281. ISBN 0-299-09430-8. \$30.

The Remarkable Reverend Clarke: The Life and Times of the Father of Australian Geology. By E. GRAINGER. Oxford University Press: 1983. Pp.292. ISBN 0-19-554365-3. £21.

Anthropology

Bison Kills and Bone Counts: Decision Making by Ancient Hunters. By J.D. SPETH. University of Chicago Press: 1983. Pp.227. Hbk ISBN 0-226-76948-8; pbk ISBN 0-226-76949-5. £16.

Excavations at Nichoria in South-West Greece, Vol.3: Dark Age and Byzantine Occupation. W.A. McDONALD et al. (eds). University of Minnesota Press: 1983. Pp.527. ISBN 0-8166-1140-0. \$49.50.

Prehistoric Times. Readings from Scientific American. Introduction by B.M. FAGAN. W.H. Freeman: 1983. Pp.262. Hbk ISBN 0-7167-1490-6; pbk ISBN 0-7167-1491-4. Hbk £22.50; £10.95.

General

About the Top Most Mysteries. By G.N. SINGH and N.D. SINGH. India: 1983. Pp.63. Pbk Rs 10.

Acoustic Emission. J.R. MATTHEWS (ed.). Gordon & Breach: 1983. Pp.167. Pbk ISBN 0-677-16490-4. \$39.50.

Advances in Materials Characterization. Materials Science Research, Vol.15. D.R. ROSSINGTON, R.A. CONDRADE and R.L. SNYDER (eds). Plenum: 1983. Pp.680. ISBN 0-306-41347-7. \$89.50.

Agricultural Soil Mechanics. Advanced Series in Agricultural Sciences, Vol.13. By A.J. KOOLEN and H. KUIPERS. Springer-Verlag: 1983. Pp.241. ISBN 3-540-12257-5/0-387-12257-5. DM 128, \$50.90.

Amazonia: Agriculture and Land Use Research. S.B. HECHT (ed.). Centro Internacional de Agricultura Tropical, Colombia: 1982. Pp.428. Pbk ISBN 84-89206-13-9. Np.

Applications de la Télédétection à l'Agro-Météorologie Opérationnelle dans les Pays Semi-Arides. European Space Agency: 1983. Pp.70. Pbk FF 60.

The Archaeology of Beekeeping. By E. CRANE. Duckworth: 1983. Pp.360. ISBN 0-7156-1681-1. £25.

Automatic Natural Language Parsing. K.S. JONES and Y. WILKS (eds). Ellis Horwood/Halsted: 1983. Pp.208. ISBN 0-85312-621-6/0-470-27460-3. £19.50, \$33.75.

Biographical Memoirs of Fellows of The Royal Society, Vol.29. The Royal Society: 1983. Pp.676. ISBN 0-85403-217-7. Np.

Coated Grains. T.M. PERYT (ed.). Springer-Verlag: 1983. Pp.655. ISBN 3-540-12071-8/0-387-12071-8. DM 140, \$55.60.

Concept Development and the Development of Word Meaning. Springer Series in Language and Communication, Vol.12. TH. B. SEILER and W. WANNENMACHER (eds). Springer-Verlag: 1983. Pp.348. ISBN 3-540-12251-6/0-387-12251-6. DM 54, \$21.50.

Conceptual Structures: Information Processing in Mind and Machine. By J.F. SCOWA. Addison-Wesley: 1984. Pp.481. ISBN 0-201-14472-7. £15.95.

Cost-Benefit Analysis, 2nd Edn. By D.W. PEARCE. Macmillan Press, London: 1983. Pp.112. Hbk ISBN 0-333-36400-7; pbk ISBN 0-333-35281-5. Hbk £12.95; pbk £3.95.

Data for Science and Technology. P.S. GLAESER (ed.). Elsevier Biomedical/North-Holland: 1983. Pp.350. ISBN 0-444-86668-X. Dfl. 125, \$49.

A Desert Country Near the Sea: A Natural History of the Cape Region of Baja California. By A. ZWINGER. Harper & Row: 1983. Pp.399. ISBN 0-06-015208-7. \$24.95.

Doomsday: Britain after Nuclear Attack. By S. OPENSHAW, P. STEADMAN and C. GREENE. Basil Blackwell: 1983. Pp.296. Hbk ISBN 0-631-13393-3; pbk ISBN 0-631-13394-1. Hbk £15.50; pbk £4.95.

The Drug User: Personality Issues, Factors and Theories. By S. EINSTEIN. Plenum: 1983. Pp.208. ISBN 0-306-40913-5. \$75.

Electric Power System Dynamics. By Y.-N. YU. Academic: 1983. Pp.255. ISBN 0-12-774820-2. \$36.50.

Equilibria, Nonequilibria and Natural Waters. Vol.1. By R.M. PYTKOWICZ. Wiley: 1983. Pp.351. ISBN 0-471-86192-8. £40.95, \$57.45.

Equilibria, Nonequilibria, and Natural Waters. Vol.2. By R.M. PYTKOWICZ. Wiley: 1983. Pp.353. ISBN 0-471-89111-8. £40.95, \$57.45.

Ferrites for Inductors and Transformers. By E.C. SNELLING and A.D. GILES. Pergamon: 1983. Pp.167. ISBN 0-86380-003-3/0-471-90208-X. £21.95, \$37.90.

Government and Research: The Rothschild Experiment in a Government Department. By M. KOGAN and M. HENKEL. Heinemann Educational: 1983. Pp.196. ISBN 0-435-82508-9. £18.50.

The Great Mental Calculators: The Psychology, Methods and Lives of Calculating Prodiges, Past and Present. By S.B. SMITH. Columbia University Press: 1983. Pp.374. ISBN 0-231-05640-0. \$39.

A Guide to Simulation. By P. BRATLEY, B.L. FOX and L.E. SCHRAGE. Springer-Verlag: 1983. Pp.383. ISBN 0-387-90820-X/3-540-90820-X. DM 72, \$28.

Imagery, Vol.3: Theoretical and Clinical Applications. J.E. SHORR et al. (eds). Plenum: 1983. Pp.435. ISBN 0-306-41464-3. \$42.50.

The Independent Nuclear State: The United States, Britain and the Military Atom. By J. SIMPSON. Macmillan Press, London: 1983. Pp.340. ISBN 0-333-23830-3. £25.

An Introduction to APL. By S. POMMIER. Cambridge University Press: 1983. Pp.136. Hbk ISBN 0-521-24977-5; pbk ISBN 0-521-27109-6. Hbk £15, \$29.95; pbk £6.50, \$11.95.

Ion-Selective Electrodes, 2nd Edn. By J. KORYTA and K. ŠTULÍK. Cambridge University Press: 1983. Pp.217. ISBN 0-521-23873-0. £25, \$49.50.

Logical and Combinatorial Algorithms for Drug Design. By V.E. GOLENDER and A.B. ROZENBLIT. Research Studies Press/Wiley: 1983. Pp.289. ISBN 0-86380-006-8/0-471-90266-7. £31, \$51.75.

Medical Care, Morbidity and Costs: Graphic Presentations of Health Statistics. By A. MIZRAHI, A. MIZRAHI and S. SANDIER. Pergamon: 1983. Pp.190. Hbk ISBN 0-08-031295-0; pbk ISBN 0-08-027082-4. Hbk £23, \$35; pbk np.

Mixed Memoirs. By G.C. THOMPSON. Paradigm Press: 1983. Pp.346. ISBN 0-9506104-2-9. £15.

Mobile Source Emissions Including Polycyclic Organic Species. D. RONDA, M. COOKE and R.K. HARCZ (eds). Reidel: 1983. Pp.387. ISBN 90-277-1633-1. Dfl 130.

A New Approach to Scientific Computation. U.W. KULISCH and W.L. MIRANKER (eds). Academic: 1983. Pp.384. ISBN 0-12-428660-7. \$39.

New Perspectives on Our Lives with Companion Animals. A.H. KATCHER and A.M. BECK (eds). University of Pennsylvania Press: 1983. Pp.588. ISBN 0-8122-7877-1. £18.75.

1984 and After: Changing Images of the Future. By N. CALDER. Century: 1983. Pp.207. ISBN 0-7126-0194-5. £9.95.

The Origins of Israel and Mankind: A Unified Cosmogonic Theory. By B. STANNARD. Carib: 1983. Pp.896. ISBN 0-9508947-0-2. £33.

Pattern Recognition Approach to Data Interpretation. By D.D. WOLFF and M.L. PARSONS. Plenum: 1983. Pp.223. ISBN 0-306-41302-7. \$29.50.

The Pharmaceutical Industry and Dependency in the Third World. By G. GEREFFI. Princeton University Press: 1983. Pp.291. ISBN 0-691-09401-2. £21.60, £8.65.

Photomasks, Scales and Gratings. By D.I. HORNE. Adam Hilger: 1983. Pp.200. ISBN 0-85274-482-X. £35.

Photomorphogenesis, Vol.16 Parts A and B. By W. SHROPSHIRE Jr and H. MOHR. Springer-Verlag: 1983. Pp.832. ISBN 3-540-12143-9/0-387-12143-9. DM 338, \$131.20.

Piaget: Critique and Reassessment. By D. COHEN. Croom Helm: 1983. Pp.163. Hbk ISBN 0-7099-0778-8; pbk ISBN 0-7099-0783-4. Hbk £31.95; pbk £6.95.

Plasma Separation and Plasma Fractionation: Current Status and Future Directions. M.J. IYAGHT and H.J. GURLAND (eds). Karger: 1983. Pp.340. ISBN 3-8055-3822-7. SwFr.134, DM 160, \$80.25.

Principles of Energetics. By K.S. SPIGLER. Springer-Verlag: 1983. Pp.168. ISBN 3-540-12441-1/0-387-12441-1. DM 68, \$26.40.

Priorities in Research. Proceedings of the 4th Symposium held May 1982, Tannus. Sir JOHN KENDREW and J.H. SHELLY (eds). Elsevier Biomedical/North-Holland: 1983. Pp.191. ISBN 0-444-90333-X. \$54, Dfl.140.

Prosody: Models and Measurements. Springer Series in Language and Communication, Vol.14. A. CUTLER and D.R. LADD (eds). Springer-Verlag: 1983. Pp.159. ISBN 3-540-12428-4/0-387-12428-4. DM 55, \$21.90.

The Psychology of Computer Use. T.R.G. GREEN, S.J. PAYNE and G.C. VAN DER VEER (eds). Academic: 1983. Pp.225. ISBN 0-12-297420-4. £12, \$19.50.

Reflections on the Universities and the National Health Service. By Sir FRED DANTON. The Nuffield Provincial Hospitals Trust: 1983. Pp.165. ISBN 0-900574-41-0. £7.50.

Relexions d'un Physicien. By A. ABRAGAM. Hermann, Paris: 1983. Pp.152. Pbk ISBN 2-7056-5960-9. FF 58.

Science of Hard Materials. R.K. VISWANADHAM, D.J. ROWCLIFFE and J. GURLAND (eds). Plenum: 1983. Pp.1,011. ISBN 0-306-41100-8. \$135.

Science and the Making of the Modern World. By J. MARKS. Heinemann Educational: 1983. Pp.507. Hbk ISBN 0-435-54780-1; pbk ISBN 0-435-54781-X. Hbk £19.50; pbk £9.50.

The Sun is Feminine: A Study of Language Acquisition in Bilingual Children. Springer Series in Language and Communication, Vol.13. By T. TAESCHNER. Springer-Verlag: 1983. Pp.246. ISBN 3-540-12238-9/0-387-12238-9. DM 125, \$49.70.

Technology and Employment in the Electronics Industry. By I. SOETE and G. DOSI. Frances Pinter: 1983. Pp.90. Pbk ISBN 0-86187-378-4. £35.

The Theory of Symmetrical Energy Structures (SES) in a Megadimensional Cosmology (MDC). Alpha Omega Research Foundations: 1983. Pp.226. ISBN 0-910122-67-9. Np.

Time Warps, String Edits, and Macromolecules: The Theory and Practice of Sequence Comparison. D. SANKOFF and J.B. KRUSKAL (eds). Addison-Wesley: 1983. Pp.382. ISBN 0-201-07809-0. Np.

Treating Child-Abusive Families: Intervention Based on Skills-Training Principles. By J.A. KELLY. Plenum: 1983. Pp.220. ISBN 0-306-41417-1. \$22.50.

Ultrasound '82. R.A. LERSKI and P. MORLEY (eds). Pergamon: 1983. Pp.638. ISBN 0-08-029805-2. £55, \$100.

The Urban Environment. By I. DOUGLAS. Edward Arnold: 1983. Pp.229. Pbk ISBN 0-7131-6392-5. £9.95.

Vegetables in the Tropics. By H.D. TINDALL. Macmillan Press, London: 1983. Pp.533. Hbk ISBN 0-333-24266-1; pbk ISBN 0-333-24268-8. Hbk £35; pbk £15.

The Victorians and Their Flowers. By N. SCOURSE. Croom Helm/Timber Press: 1983. Pp.195. ISBN 0-7099-2377-5/0-917304-89-6. £12.95.

Laboratory apparatus calling

Eleven new products are described this month including a device that 'phones home' when your experiment goes wrong.

● The Accuspec "second generation" compact gas chromatograph measures only $12\frac{1}{2}'' \times 12\frac{1}{2}'' \times 15\frac{1}{2}''$ high yet offers research-grade performance and a full range of features. The full dual column oven provides a choice of three capillary systems or $\frac{1}{8}''$ packed column facilities. Automatic cooldown is standard with the temperature programmer. Dual FID, micro-TCD and specific detectors are available.

Circle No. 140 on Reader Service Card.

● The Laser Analytics Division of Spectra-Physics Inc. has developed a cleaved-coupled-cavity Pb-salt diode laser that operates single mode over limited current and temperature ranges. The new device consists of two coupled cavity sections: the front section performs as an emitting laser and the rear section as a modulator. By varying the modulator current, it is possible to tune the emission from the front section thereby obtaining additional precise control of the laser emission characteristics.

Circle No. 141 on Reader Service Card.

● Perkin-Elmer's LC-15B is a new, low-cost, fixed wavelength UV detector for liquid chromatography. At the standard 254-nm wavelength, the maximum noise is $\pm 1 \times 10^{-5}$ absorbance units while drift is less than 2×10^{-4} absorbance units. Range settings down to around 0.0001 AUFS are included, with response times of 0.2, 0.5 and 1.0. Other features of the LC-15B include a push-button event mark, large digital readout and wide zero baseline adjustment range.

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● A high sensitivity auto-ranging electrostatic 'fieldmeter' has recently been developed by John Chubb Instrumentation of Cheltenham. The JCL 101 gives stable measurement of electric fields in the vicinity of 'static' charges on insulating or conducting surfaces.

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● Agarose IEF is a highly purified agarose optimized for isoelectric focusing by charge balancing. Gradient drift is reduced to a minimum to give high resolution, stable pH gradients and efficient isoelectric focusing. Agarose IEF is produced by Pharmacia.

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● *Ulex europaeus* agglutinin I (UEA I), a plant lectin recently claimed to be superior to antibody to factor VIII as an endothelial cell marker, is available from Vector Laboratories. UEA I has been shown to identify vascular endothelia in kidney, liver, pancreas, lung, skin, brain, placenta, umbilical cord and cultured cells as well as various tumours of endothelial origin. Unlike anti-factor VIII antibodies, UEA I can be used equally well on snap frozen or paraffin embedded tissues. Several detection systems are available including fluorescein or rhodamine conjugates for fluorescent microscopy, and affinity purified antibodies to UEA I or biotin-labelled UEA I for biotin/avidin-based histochemical techniques.

Circle No. 145 on Reader Service Card.

● The Thermex interface from Columbus Instruments converts an Apple II Plus or Apple IIe computer into an automatic printing 16-channel thermometer. As sensor, it utilizes J, K or T thermocouples. Resolution in the biological temperature range is 0.1°C . In other temperature ranges accuracy of 0.2% full-scale is achieved by individually calibrated thermocouples. A special calibration program is available to allow the user to calibrate and linearize thermocouple probes in any desired range. Thermex has built in cold junction compensation. Biological probes are available for rectal, intravenous and implantable applications for use in animals. A video monitor displays current temperatures in all channels.

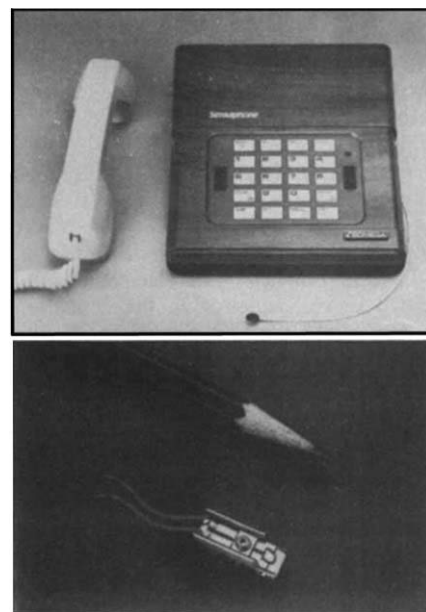
Circle No. 146 on Reader Service Card.

● An ultra-stable stirrer speed in the range 0-1,250 r.p.m. is possible with the ADI 1012 stirrer control unit. Manufactured by Applikon B.V., the controller is specially suited for use in fermentation vessels. With the aid of the ADI 1012's remote speed control input, stirrer speed can be regulated automatically with respect to the culture's oxygen content.

Circle No. 147 on Reader Service Card.

● Antibodies for the detection of oncogene protein products and related antigens — such as the epidermal growth factor receptor — are now available from Oncor. The antibodies are intended for use in immunofluorescence, immunoprecipitation and enzyme-linked assays. The range consists of: ABmyc.1, ABras.1, ABsrc.1 and AB EGF-R.1. Each antibody is supplied with sufficient product to perform 50 RIP-PAGE assays.

Circle No. 148 on Reader Service Card.



The Omega laboratory monitor (top) and Spectra-Physics new laser

● Constant process monitoring is possible with the new Omega LM-1000 laboratory monitor with voice simulator. It monitors a wide variety of process and laboratory conditions via a standard telephone jack. The LM1000 will monitor conditions such as: room air temperature or ambient sound levels — the LM-1000 can detect a process deviation alarm or presence of excess noise due to vibration. Alert conditions can be checked, for example: limit switch status on computer controlled machinery; status of valve actuated switches; pressure limit switches; and operation of solid state switching devices with control machinery. The LM-1000 automatically reacts to deviations from set conditions by phoning, in sequence, up to four pre-programmed numbers until the message is received.

Circle No. 149 on Reader Service Card.

● A new range of safety coated glass carboys in sizes up to 12 gallons is available from Wheaton Safety Co. The 'second skin' glass carboys are coated with an impact resistant, high tear thermoplastic — transparent, translucent or opaque — providing impact protection to reduce breakage potential and increasing manual handling stability. In the event of container breakage, the coating safely contains dangerous chemicals, and radioactive and biohazardous materials long enough for proper disposal.

Circle No. 150 on Reader Service Card.

These notes are based on information provided by the manufacturers. For further details circle the numbers on the Reader Service Card bound inside the journal.

Filling jobs by special initiative

Richard Pearson*

The UK Government has boasted of success in encouraging the sunrise industries. Will the manpower be there to support growth?

JOB prospects in science and technology are in a period of rapid change. While new "discoveries" are one major driving force, their application to society's needs and a changing financial context are nowadays equally strong pressures affecting employment. Existing staff need new skills, some have to retrain, while new jobs and areas of employment are always emerging. In the United States, skill shortages in information technology (IT) and electronics have led to severe depletion in the education system of the teachers needed to educate and train future specialists.

In the United Kingdom, cutbacks in the university system in the past 3 years have led many universities to rely on natural wastage to reduce staff numbers. Not surprisingly, those with marketable skills in industry have often been the ones to leave, while the rash of early retirements and freezes on recruitment have led to unbalanced staffing structures and blocked career streams. In an effort to unblock the system, a "new blood" initiative was announced by the Department of Education and Science in late 1982 which enabled the universities to recruit more than 240 new, young, teaching and research staff. There were also funds for a further 70 staff in IT as part of a wider "IT initiative" to increase the supply of graduates coming on to the labour market. Initial evaluations of these two schemes are now available.

Altogether, more than 300 posts were created in the universities and 87 per cent of them had been filled by late 1983. The University Grants Committee (UGC) reported a wide variation in the number and quality of applications for individual posts. The number of applications was as high as 80 for some posts in medicine with many strong candidates, and as low as 4 in engineering. The highest quality was found in applicants in the biological sciences and mathematics, the lowest in engineering. Information technology had the lowest number of applicants per post. Not surprisingly, the posts in engineering and information technology were hardest to fill, with nearly one in five of the information technology posts, and one in three in engineering remaining vacant (see table).

The aim of attracting young staff was largely achieved; more than half the recruits were under 30, and more than 90 per cent under the recommended age limit of 35. Interestingly, one in ten of the infor-

New posts in UGC new blood and IT initiative

Subject	Posts in 1983	Applications per post	Posts unfilled at 30 Nov. 1983
Medicine	43	19.7	5
Engineering	48	15.9	15
Biological sciences	25	32.1	3
Maths/physical sciences	86	27.3	1
Agricultural/veterinary science	8	13.7	2
Arts/social studies	32	27.5	4
Information technology	70	10.9	13
Total	312	20.8	43

mation technology recruits was over 36, while three were under 25. In a year dedicated to "Women in Science and Engineering", none of the information technology posts and only one of the engineering posts was filled by a woman, although more were appointed in medicine (6) and the sciences (9).

The IT initiative of 1982 also included additional funds for research and student places, as well as for a series of one year postgraduate and post-experience courses to increase the supply of people with IT skills. An initial evaluation of the first year of these courses, sponsored by the Science and Engineering Research Council (SERC), shows them to be making a rapid contribution to meeting recruitment needs despite their newness and a relatively low level of awareness by employers (*Manpower for Information Technology*, A. Gordon and R. Pearson, IMS). SERC is supporting two types of course, "conversion" courses aimed at graduates with no previous experience of information technology and "specialist" courses for information technology graduates.

Although many of the courses, run in both universities and polytechnics, were announced only a few months before they began in October 1982, there was a great deal of interest among first degree graduates — some course organizers received more than 300 applications for 20 or 30 places. Much of this enthusiasm was no doubt prompted by the poor job prospects for graduates without skills in information technology. Although there was a significant number of women applicants, they represented only one in eight students, possibly because many of the successful applicants had first degrees in engineering or physical science, often an essential entry requirement. The students themselves had mixed backgrounds; one in four were from polytechnics, and one in four had non-

technical first degrees. Their ages ranged from 21 to 55, half were over 25 and officially classified as "mature" students.

The numbers graduating so far, fewer than 200, were relatively low when compared with the expected 1,000 graduates in 1984. In 1983 there was little awareness among employers of this new source of skilled information technology manpower. Most employers were seeking a broad based intake of first degree graduates and it was rare to find a significant discrete demand for MSc or diploma students in any of the information technology subjects. Some were, however, sought in communications and control engineering. In the case of postgraduates with first degrees in the arts, some employers doubted whether they could learn enough useful computing in one year to be worthwhile, although one major electronics company has recently organized its own three-month courses to turn arts graduates into successful real-time programmers. By the time they graduated, most students had found jobs that used their new skills, with banks and in education as well as in the information technology industry. Starting salaries ranged from £5,000 to more than £12,000 a year, with half in the £7,000-8,000 range, well above the average starting salary for new graduates, which in 1983 was just over £6,000.

The information technology initiative has been successful so far in creating a new supply of skills. Ironically it could be poor information that constrains this supply of information technology skills in the future, with some employers unaware of these new types of graduates, and some course organizers having little interest in ascertaining industry's needs. Women still remain a large untapped source of recruits for the courses and for industry. This always assumes of course that there will be enough teaching staff to run the courses. □

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